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14. ABSTRACT

The objective of this proposal is to establish the graft preservation and immunomodulatory effects of our MP-BMPS/HBOC system in a pre-clinical large animal CTA model. We will specifically address the following specific aims in a porcine vascularized musculo-adipo-cutaneous flap model:

Aim 1: Does MP-BMPS/HBOC allow prolongation of CIT without significant cellular damage to the allograft?
Aim 2: Does the MP-BMPS/HBOC minimize the effects and incidence of ischemia-reperfusion injury at revascularization?
Aim 3: What is the effect of MP-BMPS/HBOC on the immune profile of various flap tissues after transplantation?

15. SUBJECT TERMS
Machine perfusion, limb transplantation, graft preservation, MP-BMPS/HBOC, ischemia, cold ischemia

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1. INTRODUCTION:

This study was an expansion our previous experience [1-7] with machine perfusion (MP) in combination with a newly developed hemoglobin based oxygen carrier (HBOC) solution under subnormothermic (21°C) conditions as a way to enhance organ and tissue preservation by providing effective ex-vivo oxygenation.

The experiments were performed on time and within budget. These experiments were developed as a proof-of-concept stage for the utilization of this new preservation technology in composite tissue allotransplants (CTAs).

The experiments were successful in showing the role and the effectiveness of ex-vivo oxygenation in CTAs and the superiority of this approach when compared to the cold static preservation, which is the current standard of care.

2. Key words

Machine perfusion (MP), cold static preservation (CSP), cold ischemia time (CIT), hemoglobin based oxygen carrier (HBOC), composite tissue allotransplants (CTAs), subnormothermic (SN), vascularized composite allotransplantation (VCA), vertical rectus abdominis (VRAM), University of Wisconsin solution (UW), superior epigastric artery (SEA), cytokines, metabolomics, ischemia-reperfusion injury (IRI), Belzer Machine Perfusion Solution (BMPS).

3 Summary/Specific Aims and Accomplishments

3.1. SPECIFIC AIM 1: Determine if the MP-BMPS/HBOC allows prolongation of CIT without significant cellular damage to the allograft.

This 1st specific aim was successfully achieved. We're able to show that our VRAM graft was able to be preserved for 14 hours at 21°C with no signs of any cellular damage to the allograft. The endothelial cells within the vasculature showed no signs of any damage after an extended period of time of ex-vivo perfusion. These results are comparable with previous publications from similar approaches [8-10].

The VRAM grafts showed stable weight after this extensive time of MP. The VRAM grafts show full anatomic integrity of all tissue compartments (e.g. vascular, interstitial, skin, adipose and muscular tissue). There was no damage to the vascular endothelial cells in both arterial and venous structures.

3.2. SPECIFIC AIM 2: Determine if the MP-BMPS/HBOC system minimizes the effects and incidence of I/R injury at revascularization.

The 2nd specific aim was successfully achieved. The MP/HBOC system provided effective oxygenation during the VRAM preservation. The tissues were intact and metabolically active. The magnitude of the IRI was significantly higher in the control

group (CSP), where early apoptosis was subsequently followed by necrosis in both the adipose and muscular tissues. The control group showed early contraction bands within the muscular tissue within the initial period (4 hours). These contraction bands expanded over time and led to further apoptosis and necrosis after VRAM graft reperfusion [11, 12]. The VRAM grafts perfused by the MP/HBOS system didn't show signs of contracting bands over the extended period of preservation (14 hours) and clear features of a self-sustained and mild IRI after reperfusion.

Our transplant pathologists were able to provide a more objective assessment of the IRIs by scoring them blindly within 5 different segments of the VRAM grafts (e.g. skin, subcutaneous adipose tissue, muscle, microvasculature and large hilar vessels). This modified scoring system showed a higher score of inflammation and tissue damage in the control group (CSP).

Further histological analysis is underway. Our preliminary analysis shows a lower degree of inflammation across the board in the MP, which reinforces our previous similar findings in liver allografts.

3.3. SPECIFIC AIM 3: Determine the effect of MP-BMPS/HBOC on the immune profile of various flap tissues after transplantation.

Tissues exposed to prolonged oxygen deprivation, or "ischemia", followed by restoration of blood-flow, can display a wide range of features compatible with IRI. The lack of oxygen supply to the control group would trigger the following cycle that ultimately lead to cell death. Figure 1 [13] displays the metabolic pathways involved in persistent anoxia.

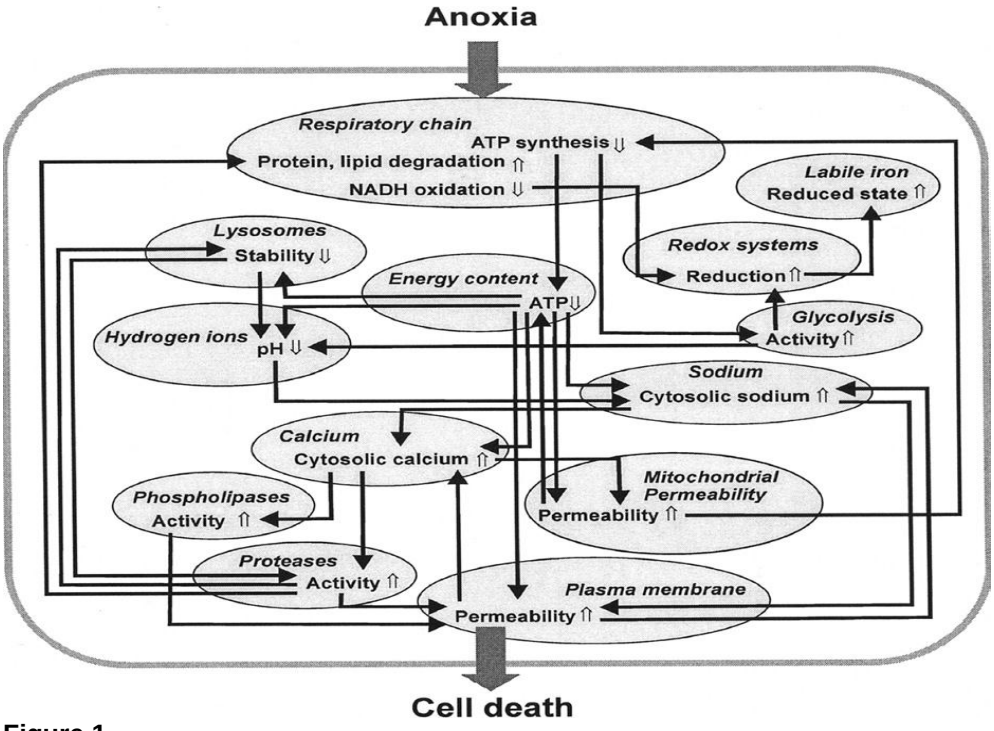


Figure 1

Tissue injury from IRI can involve both the parenchymal and the endothelial cells of the grafts being preserved ex-vivo. Figure 2 displays the pathogenic network of the inflammatory responses, where self-amplifying loops are responsible for the expansion and propagation of the immune response towards necrosis and irreversible tissue damage [13].

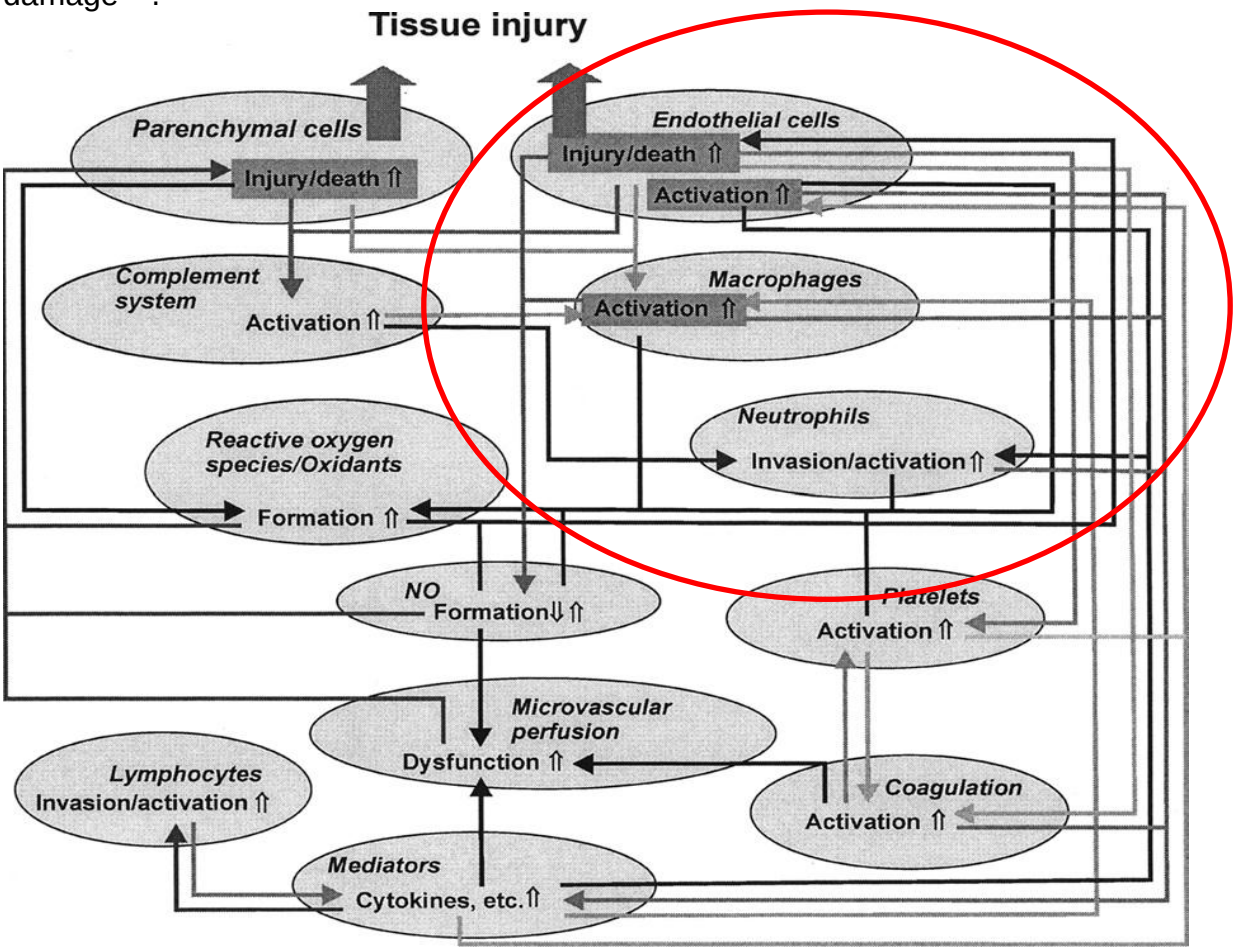


Figure 2

Our experiments revealed a significant amount of neutrophil activation after reperfusion in the CSP, where a more pronounced inflammatory response was observed when compared to the MP group. In the CSP, the restoration of blood flow after graft implantation appeared to trigger the activation of endothelial cells, which was followed by enhanced adhesion of neutrophils and platelets to the vascular endothelium. This led into increased microvascular permeability and perivascular leakage. Furthermore, a significant amount of macrophages were seen in the CSP group. The macrophages were further differentiated into histiocytes and played a significant role in the formation of calcified granulomas (calcinosis) within the muscular tissue.

The overall magnitude of the recipient's inflammatory response was significantly lower in the MP group, where reliable levels of oxygenation were sustained over the preservation period (14 hours) prior to transplantation.

Based on our initial histological analysis, the use of our MP/HBOC system has a significant beneficial effect in minimizing the magnitude of the IRIs seen immediately after graft preservation. The downstream effect on the MP/HBOC system in the immune profile of the VRAM graft is also beneficial, since the initial decrease in inflammation from the preservation stage appears to minimize further cell infiltration and the irreversible tissue damage clearly observed in the control group (CSP), which were treated according to the current standard of care for clinical preservation and transplantation.

4. Accomplishments

The surgical procedures utilized in this project were successful and no technical complications were documented with the swine vertical rectus abdominis (VRAM) myocutaneous flap. All the animals received triple immunosuppression (Tacrolimus, Mycophenolate Mofetyl and Prednisone) and there no adverse events documented during the post-operative period (7 days). All the animals were managed within USDA guidelines within an approved IACUC protocol and there were no issues related to the animal experiments.

There were several sets of data obtained from the experiments. Table 1 shows a summary of all the groups and samples collected during both stages.

Group	Intervention	Description
1. Ex Vivo Control (n=4) CSP Standard of Care	4 VRAM grafts preserved for 14 hours with UW at 4°C	Control group – baseline studies
2. Ex Vivo Study Group (n=4) Machine Perfusion	4 VRAM grafts preserved for 14 hours with MP at 21°C	Study group – developments for machine perfusion device
3. In Vivo Control (n=4) CSP Standard of Care	4 VRAM grafts preserved for 14 hours with UW at 4°C and transplanted heterotopically (cervical)	Control group – baseline for ischemia reperfusion injuries and VRAM graft viability after 7 days
4. In Vivo Study Group (n=4) Machine Perfusion	4 VRAM grafts preserved for 14 hours with MP at 21°C and transplanted heterotopically (cervical)	Study group – impact of MP in ischemia reperfusion injuries and VRAM graft viability after 7 days

Table 1

The VRAM flap recovery techniques were uneventful. The VRAM graft has medium-caliber vessels (artery and vein) feeding a sizable portion of tissue containing skin,

subcutaneous adipose tissue and muscle. All VRAM grafts were flushed with cold (4°C) UW solution after cross clamp.

Figure 3. Schematic representation of the anatomical features of VRAM grafts in humans. Our experiments were based on the recovery and transplantation of the VRAM grafts though perfusion by the superior epigastric artery (SEA) vascular pedicle.

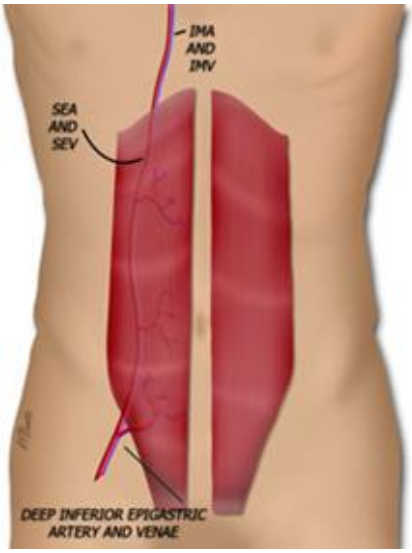


Figure 3

Figure 4. The VRAM graft outlined in the donor before graft procurement.

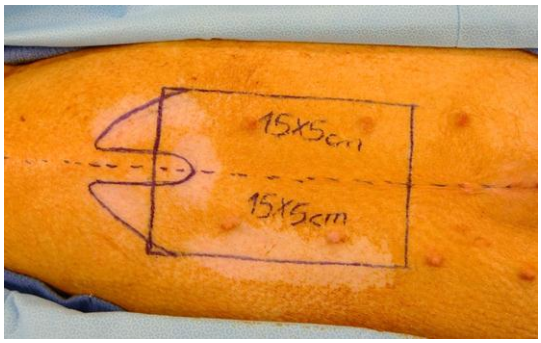


Figure 4

Figure 5. Completion of the surgical procedure to recover the VRAM graft in our swine model. The ruler is aligned with the completely isolated pedicle of the SEA.

Figure 5

These preservation and transplantation experiments with the VRAM graft were conducted in 2 separate stages. Step 1 was named ex-vivo and primarily focused on preservation and in the technical features (e.g. pressures, flow, oxygenation, temperature) involved in MP.
Step 1 – Ex-vivo stage:

Ex-vivo experiments with the VRAM grafts were conducted over a 14 hour preservation period. VRAM grafts were surgically recovered and preserved by our MP/HBOC system (n=4) while being compared to cold static preservation (CSP) (n=4) as the current standard of care.

The ex-vivo experiments were devoted to streamline all the technical aspects related to VRAM graft perfusion, since this was a new application of our technology previously and successfully performed in both porcine and human liver allografts.

Machine Perfusion of the skin flaps was performed using our initial prototype Organ Assist Liver Assist® device in combination with our HBOC solution, which contains a hemoglobin-based oxygen carrier and organ preservation solution mixed 1:3 ratio, respectively. The starting hemoglobin as measured by an ABL800flex (Radiometer, Copenhagen) blood gas analyzer was 3.4 g/dL. The baseline settings for the MP system were: 60 mmHg pressure, 21°C, FiO2 60%, sweep gas 0.3 L/min. Perfusion was initiated with an inlet pressure of 60mmHg at 1Hz pulse pressure, achieving a flow of ~10mL/min. Initial blood gas values were ~93% saturated HBOC solution at a pO_2 of ~400 mmHg. As the initial vasoconstriction from VRAM recovery subsided, the flows progressively increased over time. The MP device alters centrifugal pump speed to maintain a set pressure. After 2 hours, with flows exceeding 25ml/min, the pressure set point was lowered to 50 mmHg where it was maintained throughout the remainder of the perfusion. After 14 hours, the VRAM graft was removed from the MP device, weighted and processed for additional studies.

Step 2 - In-vivo stage – VRAM graft transplantation and the assessment of the IRIs after preservation by 2 different methods (CSP vs. MP)

The in-vivo studies were also composed by 2 groups of 4 animals each. The study group (MP) had 4 animals transplanted heterotopically (cervical implantation using the

carotid artery for inflow and the external jugular vein for venous drainage) with the VRAM allografts after a period of 14 hours of preservation where full oxygenation was provided within a low pressure and low flow protocol. The control group (CSP) had 4 animals transplanted with VRAM allografts after 14 hours of preservation with CSP as the current standard of care. Both groups were followed for 7 days. The VRAM grafts were biopsied on days 2, 4 and 7. An end-study necropsy was performed on day 7. All the animals received full immunosuppressive therapy composed by Tacrolimus, Mycophenolate Mofetil and Prednisone. All the animals had daily clinical and laboratorial assessment. Additional studies (e.g. transcriptomics, proteomics and metabolomics) were performed to assess graft viability and the impact of ischemia reperfusion injuries (IRI) suffered after this prolonged period of preservation.

Final Results from the MP experiments

Perfusion flow rates

The VRAM grafts were properly perfused with low flows and showed no signs of weight gain or endothelial cell damage during the extended time (14 hours) of MP. Figure 6 displays the VRAM graft on the PVC mesh during MP.

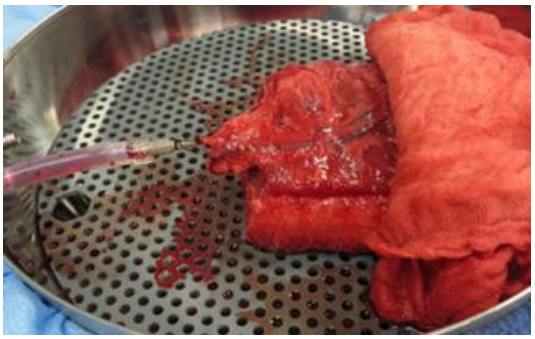


Figure 6

Figure 7 displays the flow rates (ml/min).

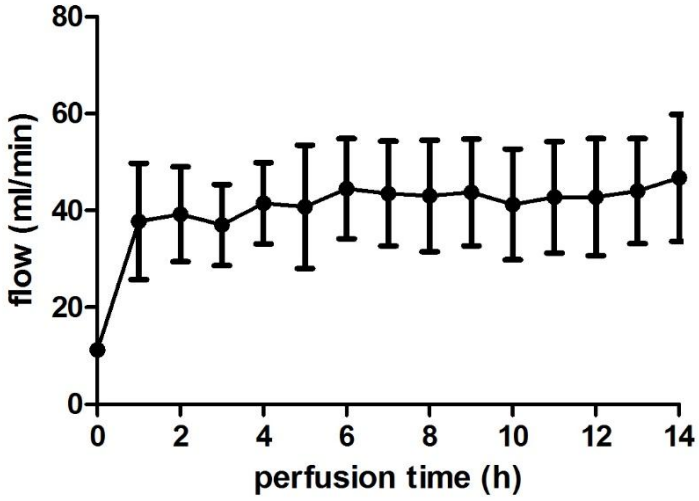


Figure 7

Perfusion pressures

The MP device was able to provide reliable pressures over the 14 hour perfusion protocol. The system was developed to provide lower than physiological arterial pressures as a way to sustain endothelial cell integrity within the macro and microvasculature. Figure 8 displays the pressures (mm/Hg) over the 14 hour period.

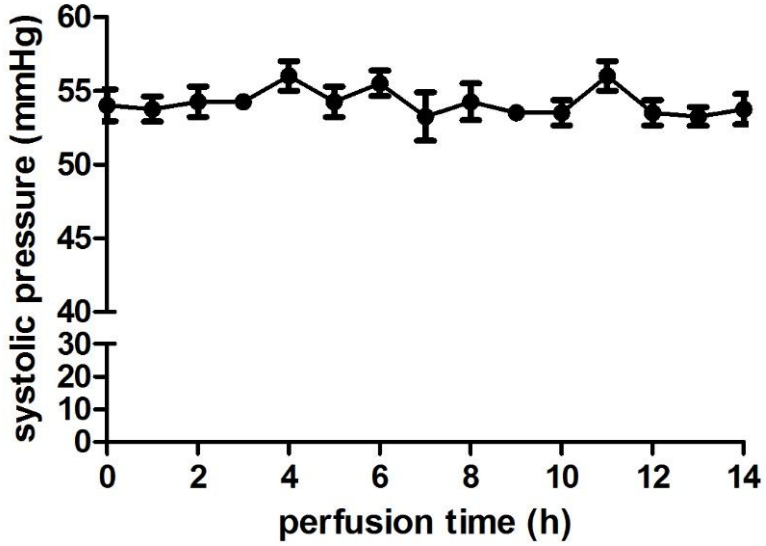


Figure 8

Oxygen delivery

The MP system in combination with our previously developed HBOC was capable to deliver adequate amounts of oxygen to the VRAM grafts over a 14 hour period at 21°C. Figure 9 displays oxygen (O_2) delivery (mlO_2/min/g tissue) over a 14 hour period.

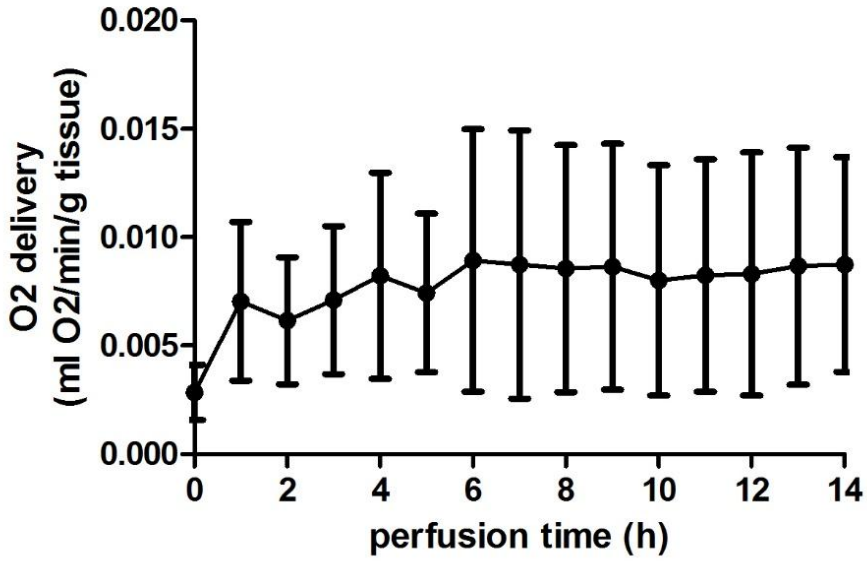


Figure 9

Indirect measurements of O_2 delivery were also assessed by measuring lactate concentration within the perfusate. Figure 10 displays lactate levels (mmol/L) in the perfusate over a 14 hour period at 21°C.

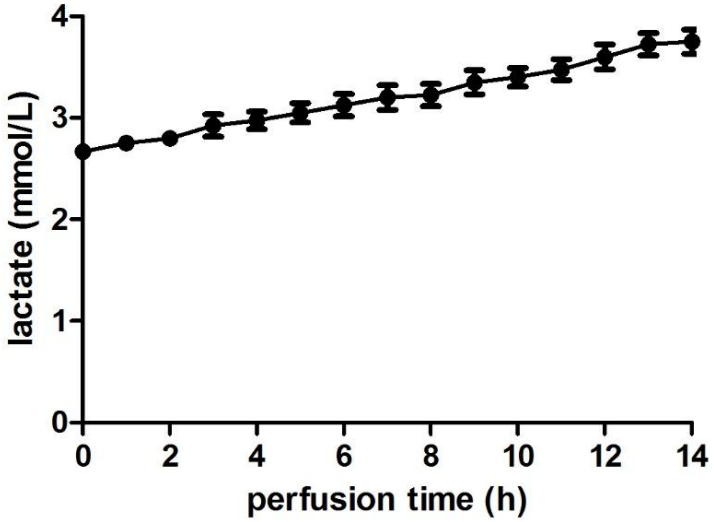


Figure 10

Figure 11 displays the pH continuously monitored in the perfusate during the MP protocol.

There were no signs of acidosis and no NaHCO3 infusions were required to sustain a physiologic (7.35-7.45) pH over a 14 hour period.

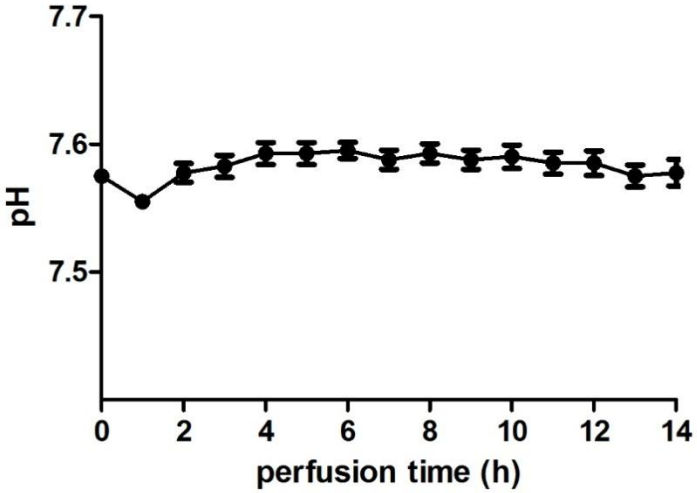


Figure 11

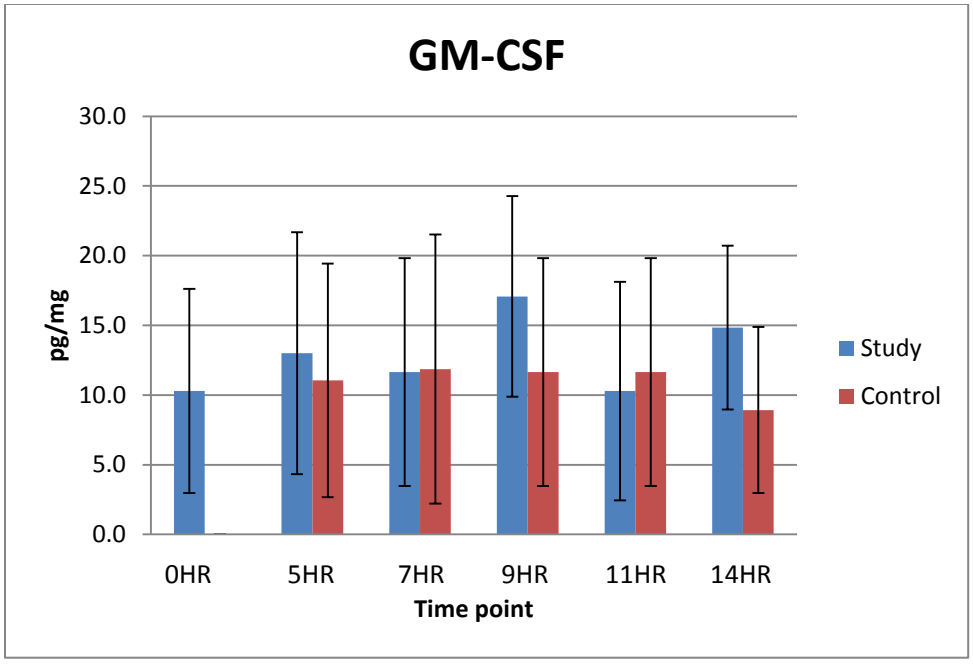
Inflammatory markers

In order to quantify the inflammatory process triggered by IRI and the imminent alloreaction against the VRAM after graft implantation, a full cytokine profile was obtained during VRAM graft preservation in both groups (MP and CSP). Subsequent samples were obtained from tissue biopsies during the post-operative period.

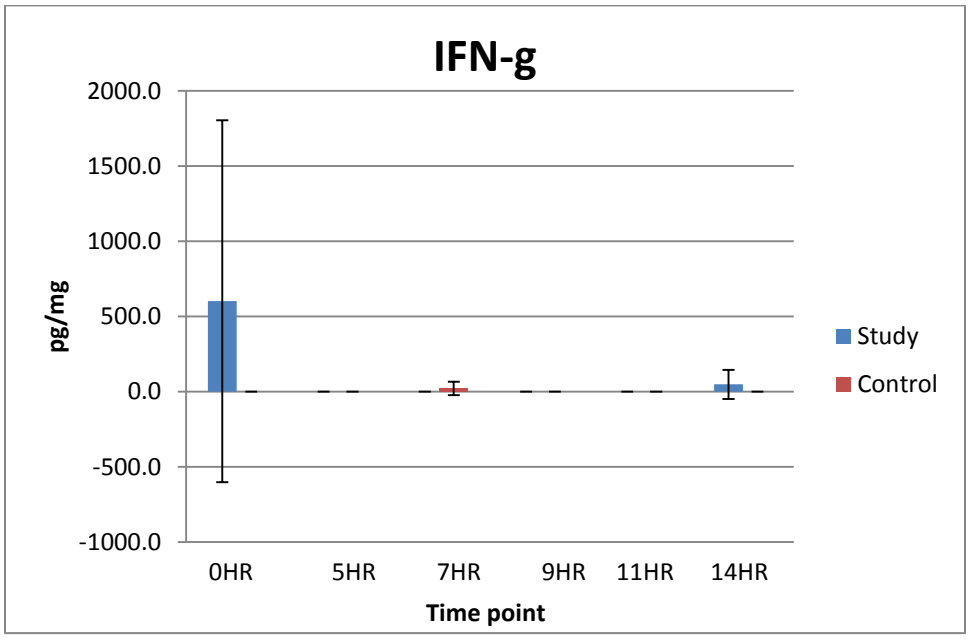
Tissue and perfusate assays of interferon IFN-γ, IL-10, IL-12/IL-23 p40, IL-1β, IL-4, IL-6, IL-8 and tumor necrosis factor (TNF)-α were carried out using a Luminex™ beadset from Affymetrix (Santa Clara, CA). GM-CSF, IL- 1α, IL-1RA, IL-2 and IL-18 were measured using a Luminex™ beadset from Millipore (Merck KGaA, Darmsdadt, Germany)[28]. Perfusate samples were also assessed for arterial blood gases (MP group only) and biochemical parameters. Tissue samples were normalized by protein content to account for experimental variability in cell number and protein concentration among individual samples.

The results during the ex-vivo stage (graft preservation) were the following:

1. GMCSF



2. IFN-g


IFN-g
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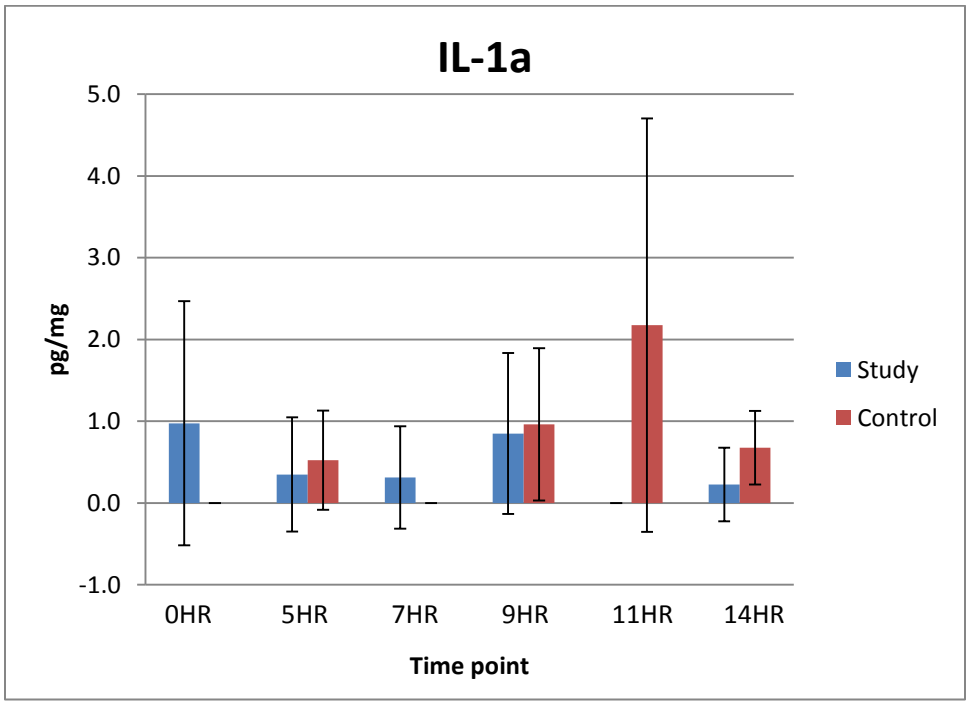
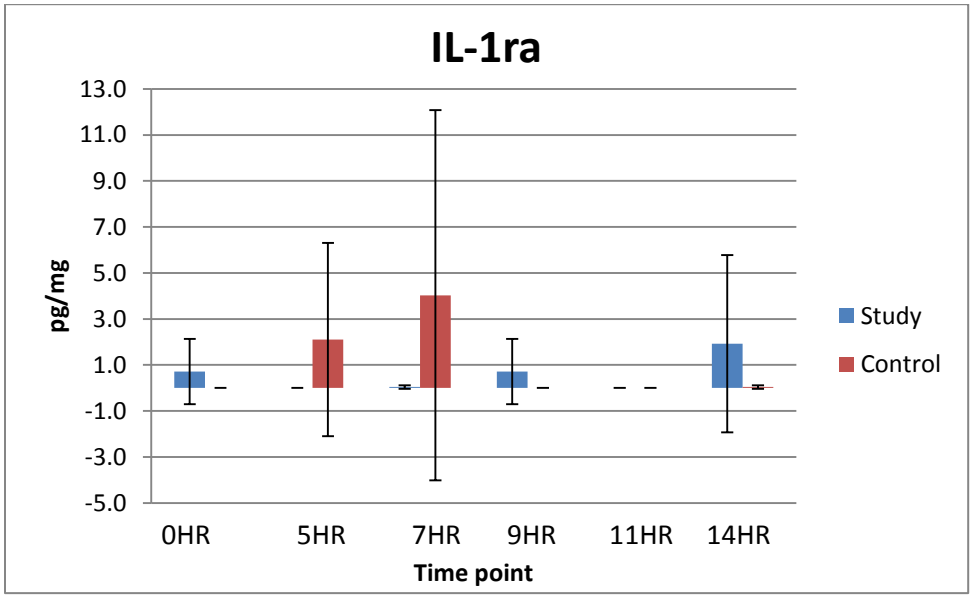
1500.0
1000.0
500.0
0.0
-500.0
-1000.0
pg/mg
0HR
5HR
7HR
9HR
11HR
14HR
Time point
Study
Control

3. IL-1a


IL-1a
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
pg/mg
0HR
5HR
7HR
9HR
11HR
14HR
Time point
Study
Control

4. IL-1ra


IL-1ra
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11.0
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7.0
5.0
3.0
1.0
-1.0
-3.0
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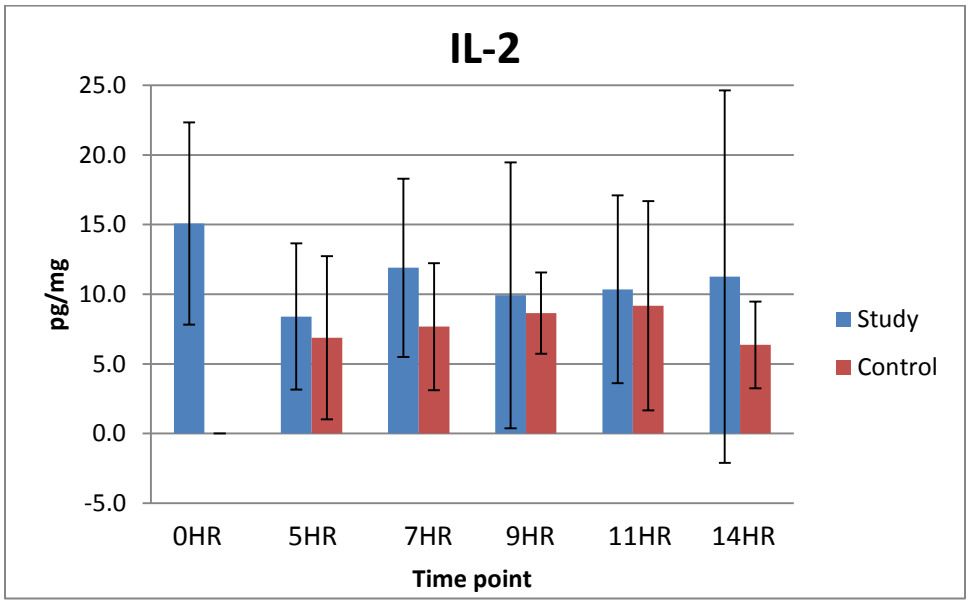
9HR
11HR
14HR
Time point
Study
Control

5. IL-2


IL-2
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20.0
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10.0
5.0
0.0
-5.0
pg/mg
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5HR
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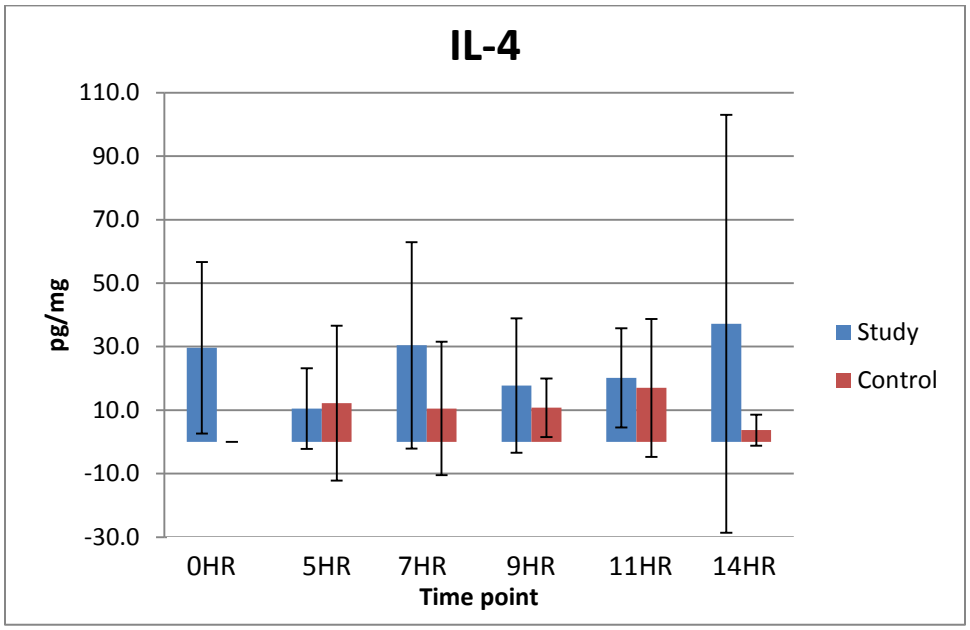
14HR
Time point
Study
Control

6. IL-4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10.0
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pg/mg
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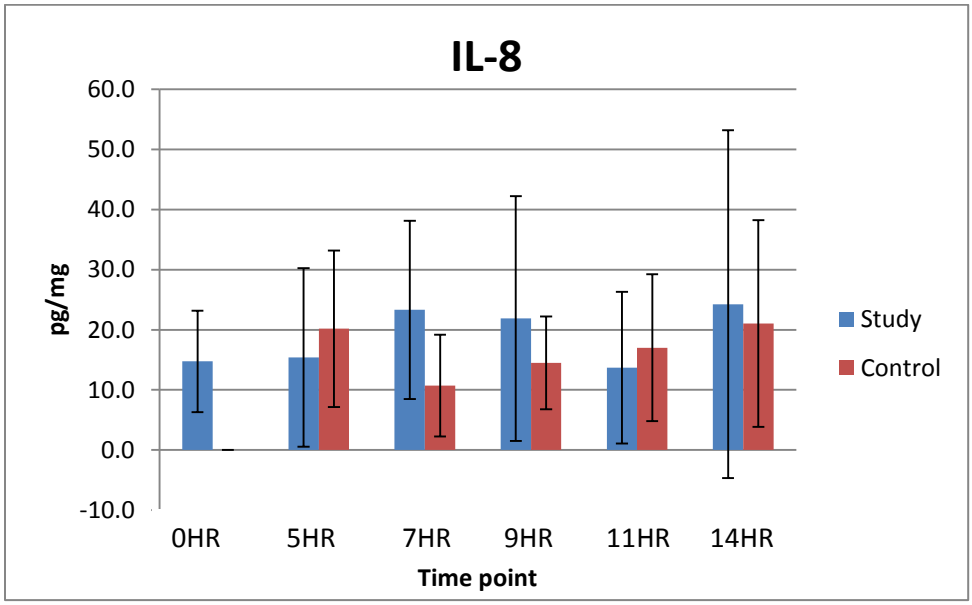
11HR
14HR
Time point
Study
Control

7. IL-8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pg/mg
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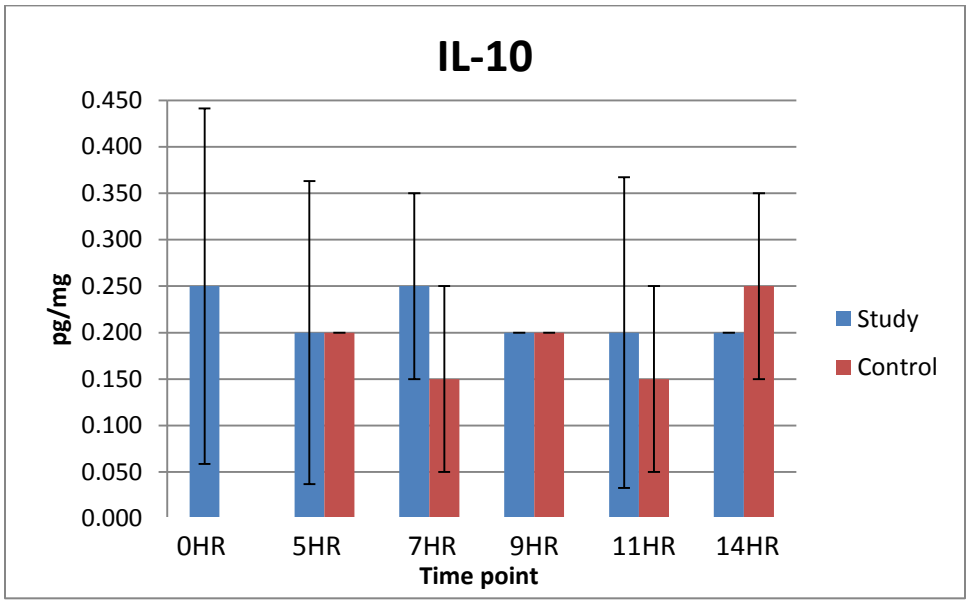
Time point
Study
Control

8. IL-10


IL-10
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0.100
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0.000
pg/mg
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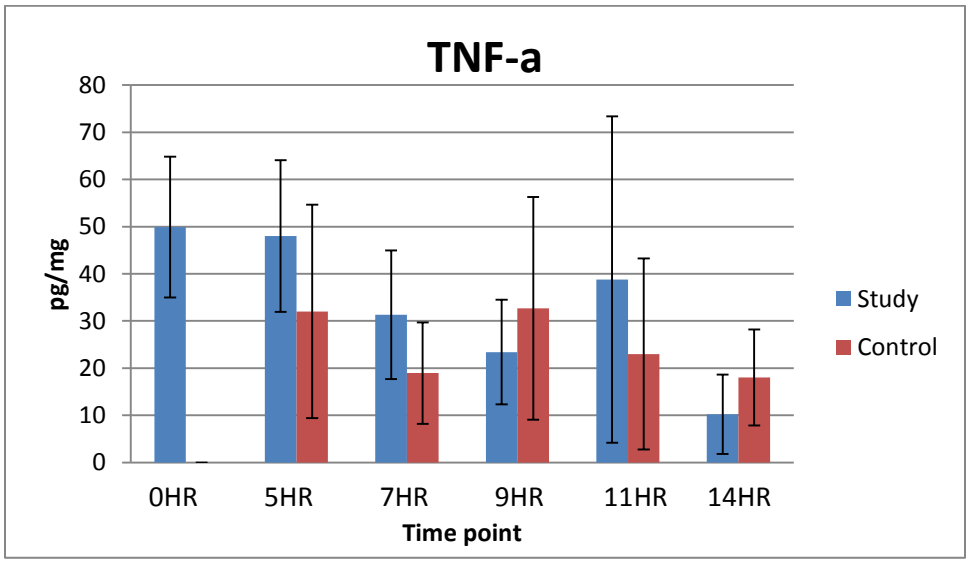
9HR
11HR
14HR
Time point
Study
Control

9. TNF-α


TNF-a
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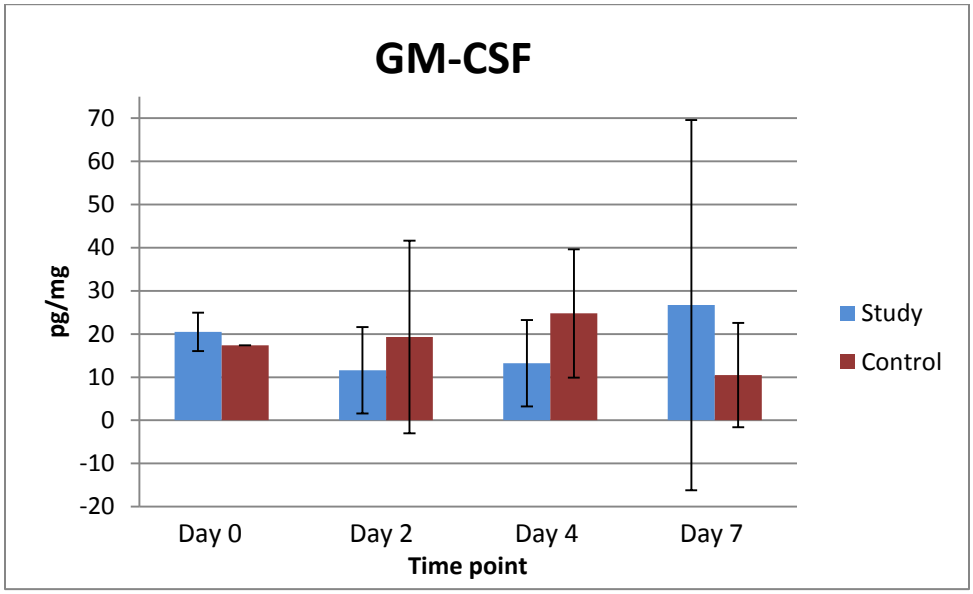
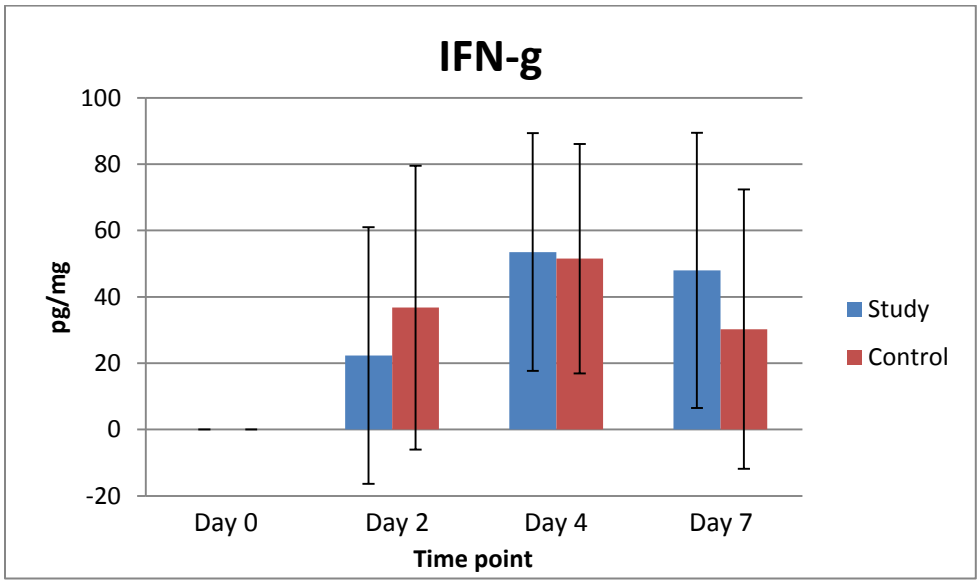
70
60
50
40
30
20
10
0
pg/mg
0HR
5HR
7HR
9HR
11HR
14HR
Time point
Study
Control

The results during the in-vivo stage (after transplantation) were the following:

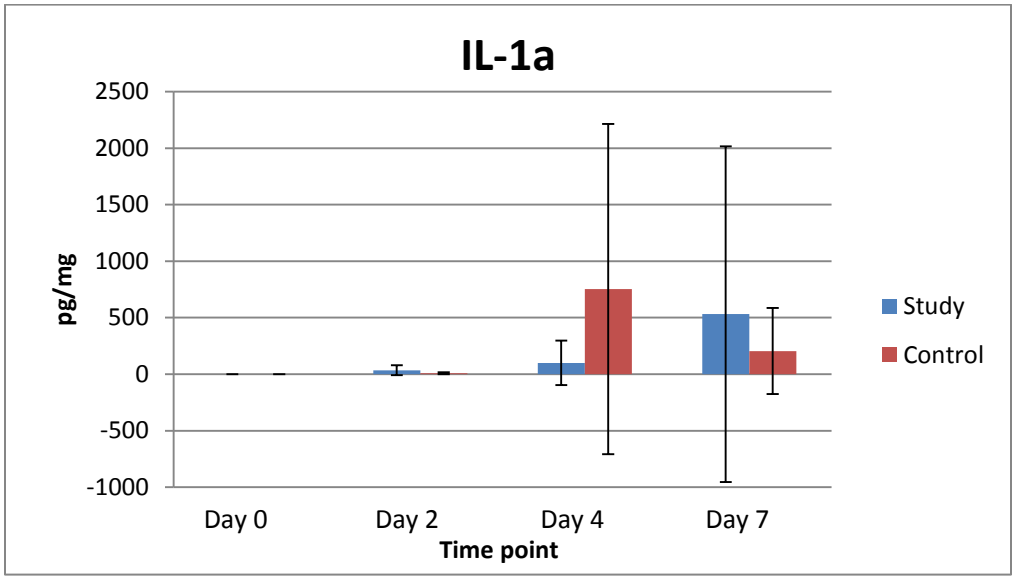
1. GM-CSF



2. IFN-g



3. IL-1a


IL-1a
2500
2000
1500
1000
500
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-500
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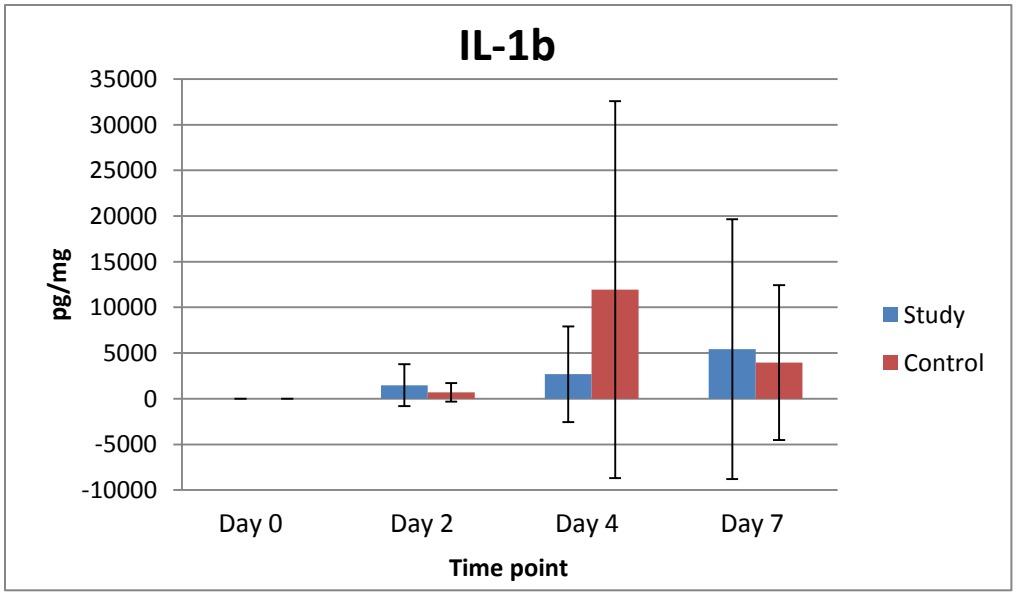
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

4. Il-1b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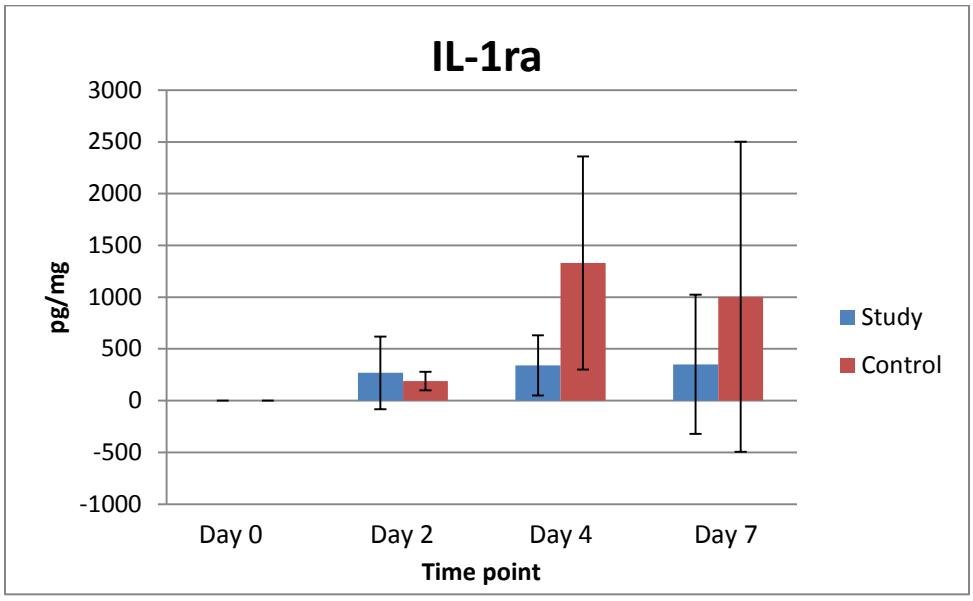
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

5. IL-1ra


IL-1ra
3000
2500
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1500
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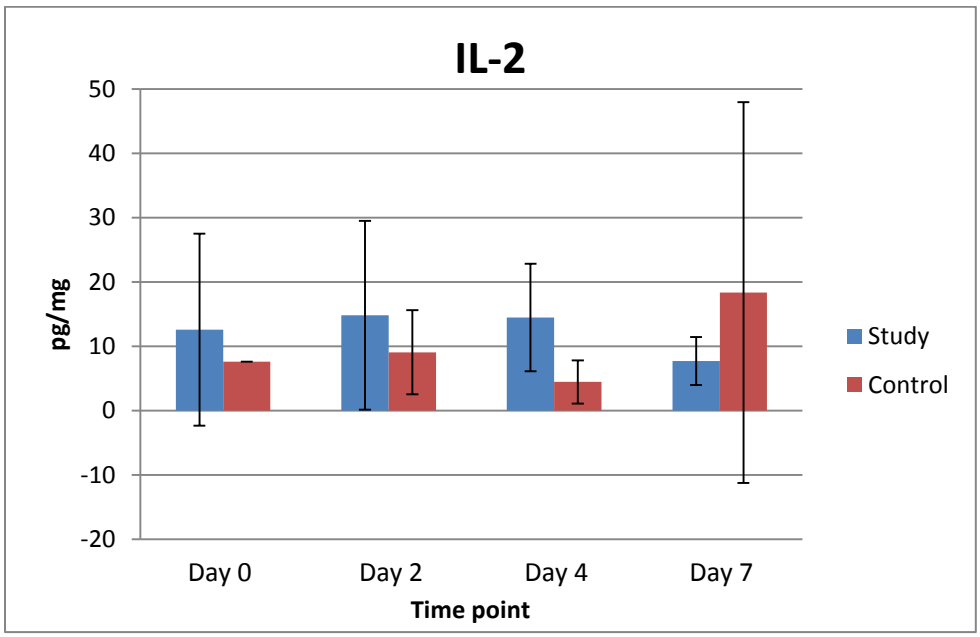
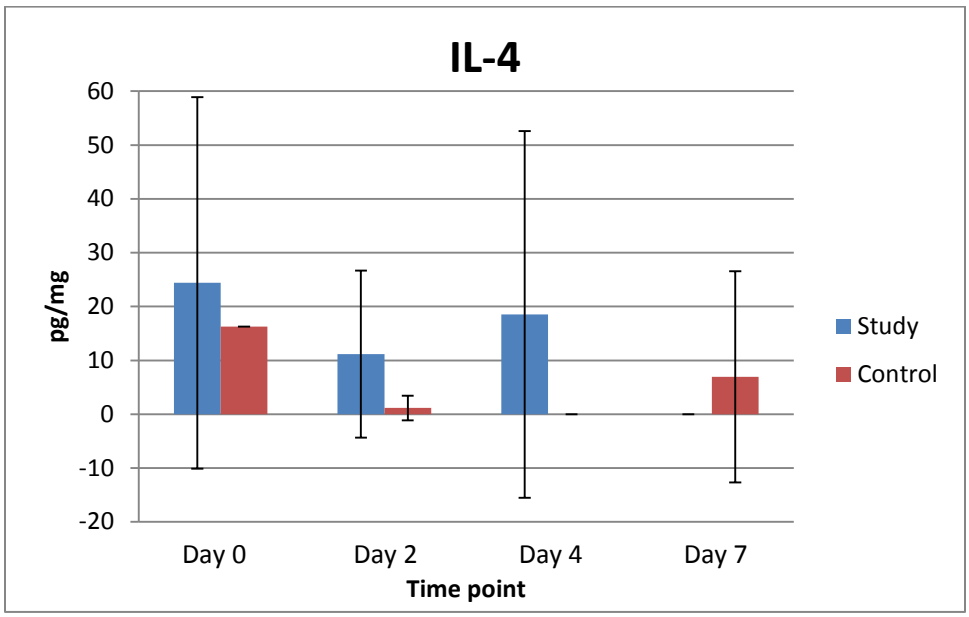
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

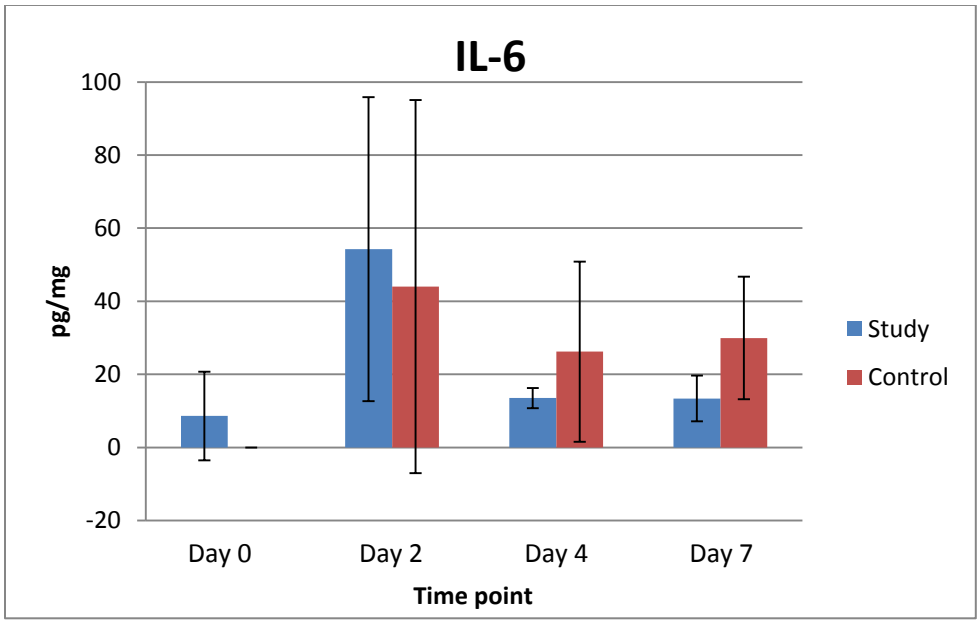
6. IL-2


IL-2
50
40
30
20
10
0
-10
-20
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

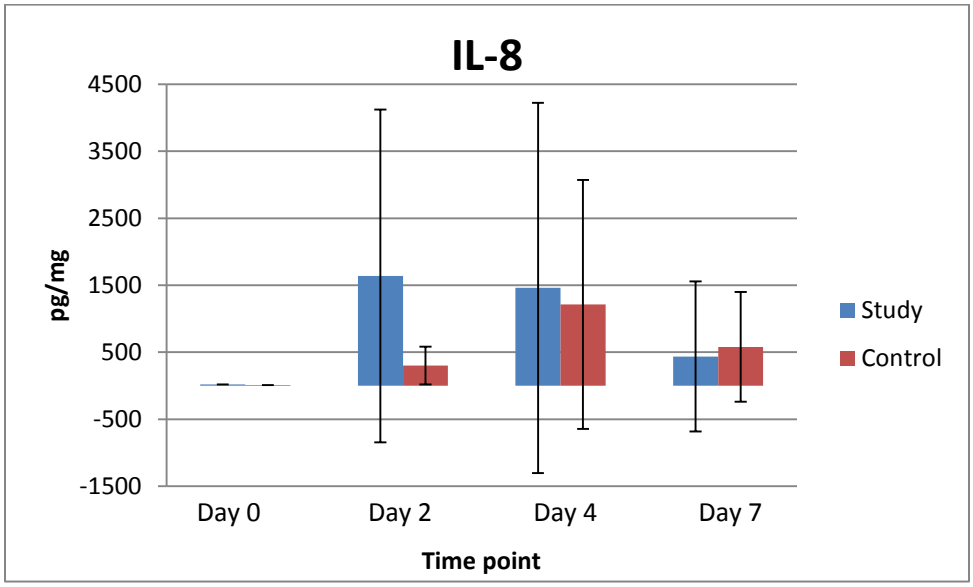
7. IL-4


IL-4
60
50
40
30
20
10
0
-10
-20
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

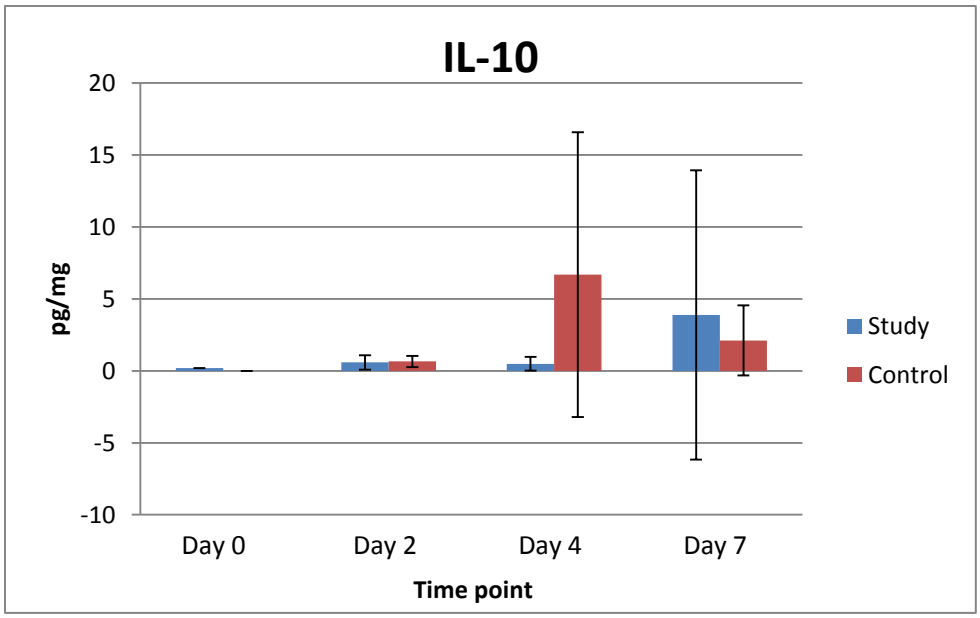
8. IL-6


IL-6
100
80
60
40
20
0
-20
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

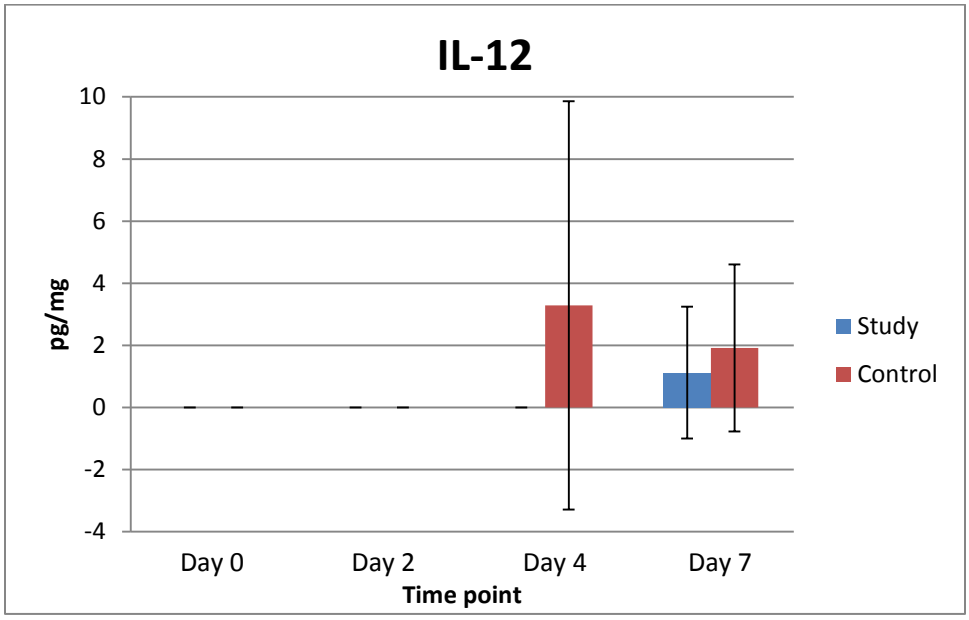
9. IL-8


IL-8
4500
3500
2500
1500
500
-500
-1500
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

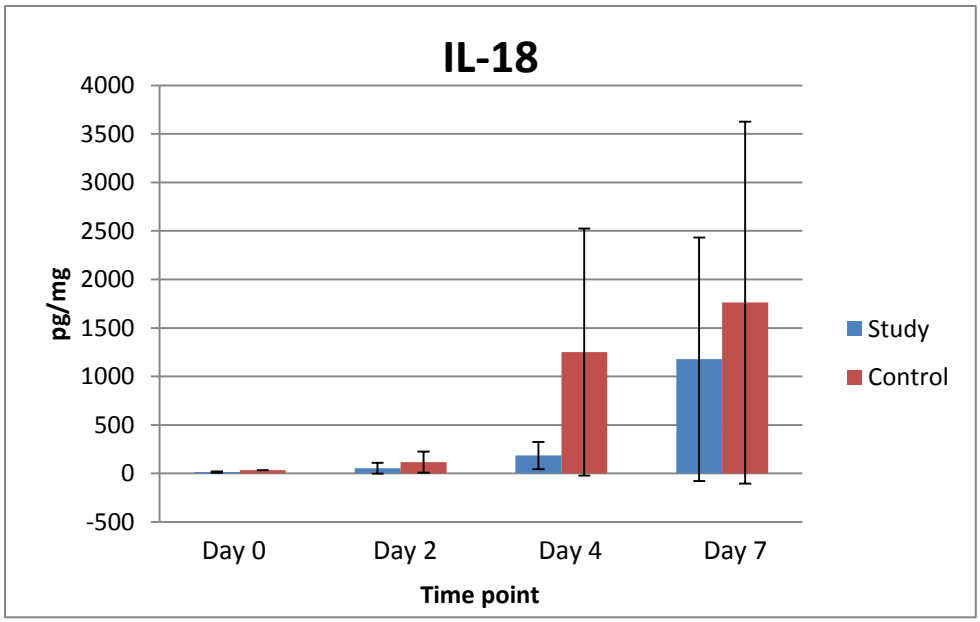
10. IL-10


IL-10
20
15
10
5
0
-5
-10
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

11. IL-12



12. IL-18



Clinical results

The transplant recipients received triple immunosuppressive (IS) therapy (Tacrolimus, Mycophenolate Mophetyl and Prednisone) after a single dose of Solumedrol (1g) was given intraoperatively prior to graft reperfusion. A central venous access (Broviac

catheter) was placed at the end of the surgical procedure to assure continuous venous access throughout the entire post-operative period.

The VRAM grafts were inspected daily and punch biopsies were performed on days 2, 4 and 7. The end-study necropsy was conducted on post-operative day 7 after the animals have been properly euthanized.

Clinical assessment of the VRAM grafts revealed normal features in the recipients of the MP group. The CSP animals showed signs of progressive ischemic changes (e.g. shallow ulcerations) within the graft. These shallow ulcers evolved into full ulceration of the nipples by day 7 (See figure 12).

There were no technical complications from all the surgical procedures and the animals tolerated the IS therapy well with no major adverse events.

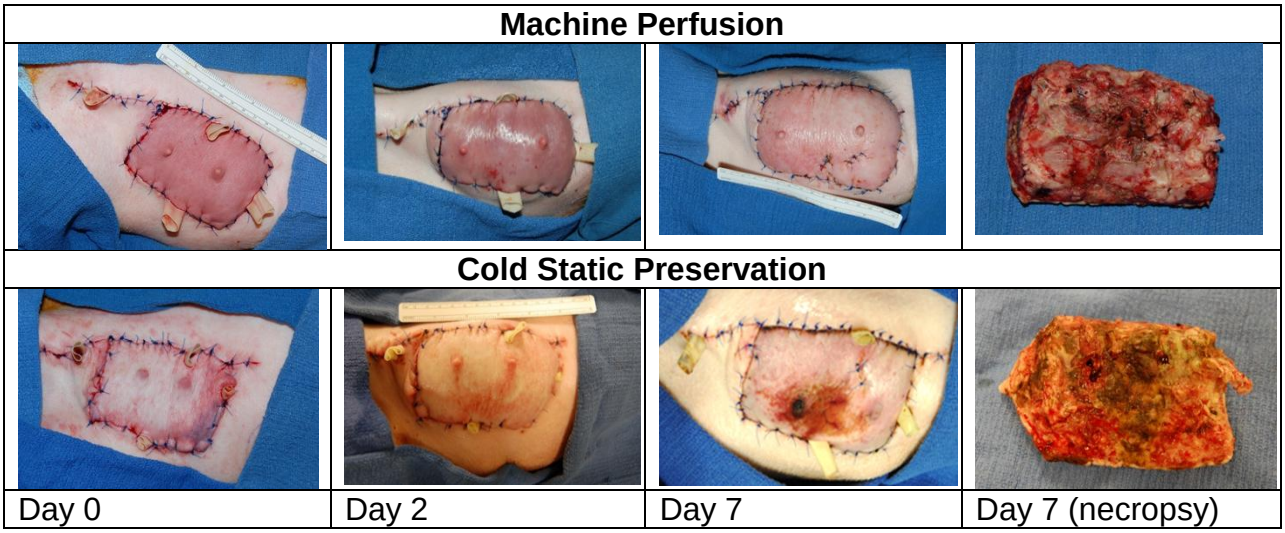


Figure 12

Myoglobin levels in the recipient's peripheral blood after transplantation

In order to assess the IRI and the level of VRAM damage after 14 hours of preservation on both groups, myoglobin levels were measured daily over a 7 day period. Figure 13 displays the data collected over a 7 days period on both groups of animals (CSP and MP).

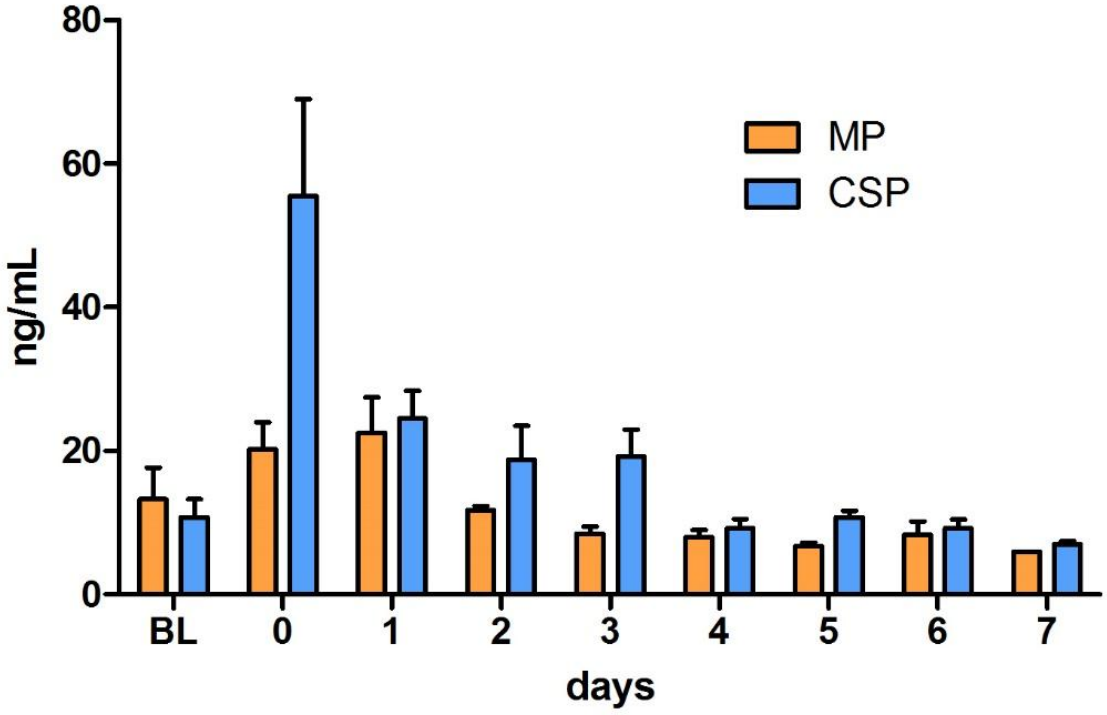


Figure 13
Histological analysis and scores for IRIs

*** p < 0.05 vs machine perfusion**
 # p < 0.05 vs Baseline (BL)
n=4 each group

A transplant pathologist who was not involved with the initial experiment design and further data collection performed a blind review of all the tissues biopsied throughout all phases of both protocols.

The following criteria were developed for a new IRI score, which has initially based on previous literature [8-10].

The VRAM grafts were divided in 5 segments:

1. Skin and epidermis
2. Subcutaneous tissue
3. Muscular tissue
4. Large vessels (hilar structures of the VRAM graft)
5. Nerve tissue

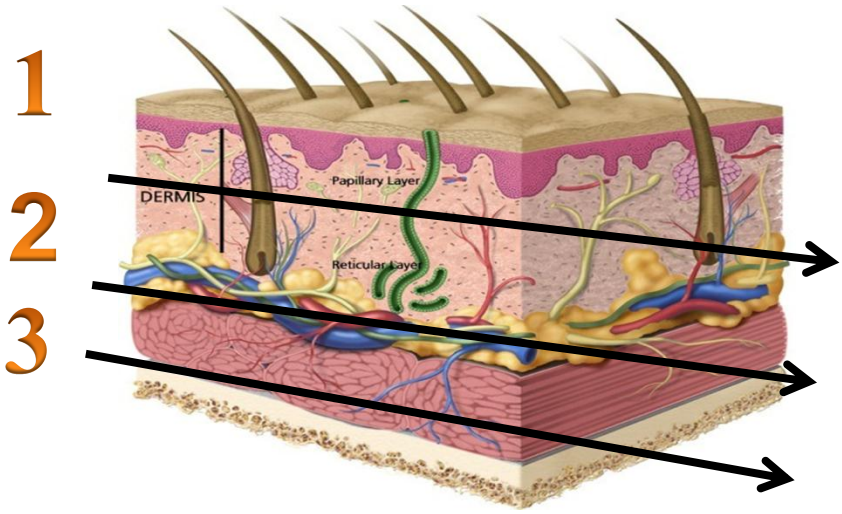


Figure 14

The following parameters were considered:

1. Tissue ischemia (e.g. contracting bands in the muscle)
2. Tissue necrosis
3. Degree of inflammation
    a. Margination of leukocytes, neutrophils and macrophages within interstitial capillaries
    b. Perivascular lymphocytic inflammation in small size vessels

A Histologic Injury Severity Score (HISS) was developed. The histological parameters analyzed within the different segments were the following:

1. Skin
    a. eosinophilic infiltration
    b. lymphocytic infiltration
    c. pyknosiskaryorrhexis
    d. karyolysis
    e. necrosis/apoptosis
2. Subcutaneous tissue
    a. perivascular infiltration
    b. lymphocytic infiltration
    c. adipocyte necrosis/apoptosis
    d. calcifications, fat necrosis
3. Muscle tissue
    a. Contraction bands (disturbed/lost cross striations)
    b. disrupted muscle fibers
    c. decomposed endomysium
    d. decomposed epimysium
    e. edema
4. Nerves
    a. intramyelinic edema
    b. endoneural edema
    c. compression induced myelin damage

    d. axonal vacuolization
    e. axotomy
5. Large hilar vessels
    a. intraluminal thrombi
    b. loss of endothelia layer small vessels
    c. loss of endothelia layer medium vessels
    d. fragmentation of internal lamina elastic
    e. fragmentation of external lamina elastic
    f. vasa vasorum involvement
    g. perivascular edema
    h. erythrocyte extravasation
    i. leukocyte adhesion
    j. leukocyte infiltration

The severity of the IRIs was scored according to the following system:

Severity of the IRI	**Score**
None	0
Mild	1
Moderate	2
Severe	3

The nerve tissue was also assessed in spite of the lack of nerve reconstruction within the VRAM graft. The assessment was mostly focused on the degree of perineural inflammation.

The hilar vessels were initially examined (gross pathology) during the end-study necropsy to rule our thrombosis stemming from potential surgical complications.
The vessels were further fixed in paraffin and sectioned for subsequent microscopic analysis.

Table 2 displays the histological classification utilized to score the IR lesions seem after reperfusion.

Score for histopathologic alterations of H&E stained sections (ref 1)	
compartment 1) Skin= epidermis + papill - retic derma	
0	tissue not affected
1	mild perivascular inflammatory infiltrate of dermal microvasculature including margination of PMN. No involvement of overlying epidermis
2	moderate perivascular Lymphocy inflammation + margination of PMN +interface inflammation with infiltration of epidermis & epithelial cell necrosis
3	severe, dense perivascular and dermal inflammation with necrosis and epiderm loss
Compartment 2: Subcutis = adipose tissue with small-medium size vessels	
0	tissue not affected
1	mild perivascular inflammatory infiltrate small size vessels including margination of PMN
2	moderate perivascular inflammation with focal adipose tissue necrosis
3	major necrosis, loss of architecture, calcinosis
comparment 3: Muscle (myocytes & intramuscle interstitial capillaries/small size vessels)	
MYOCYTES	
0	tissue not affected
1	mild ischemic changes (bands of contraction // loss of striation (and or mild, localized perivascular inflammatory infiltrate less than 50%
2	multifocal ischemic changes/necrosis and/or perivascular inflammatory infiltrate including margination of leukocytes (neutroph and macrophages) in interstitial capillaries
3	major muscle cell necrosis and calcification
interstitial inflammation /microvasculature	
0	normal
1	mild interstitial/ perivascular inflammatory infiltrate including margination of PMN.
2	moderate interstitial/perivascular Lymphocy inflammation including margination of PMN
3	severe, dense interstitial/perivascular inflammation
Nerves Histological evaluation showed non specific changes - need additional studies	
0	tissue not affected
1	mild perineural inflammatory infiltrate, mild vacuolization and mucoid degeneration
2	severe perineural inflammatory infiltrate, vacuolization and mucoid degeneration, minor alterations of nerve structure
3	severe degeneration and loss of typical nerve structure
Vessels medium and large size: Pedicle	
0	tissue not affected
1	reactive endothelial cells
2	perivascular infiltration and/or alterations in vessels architecture (lamina elastica, smooth muscle)
3	major vessel damage, loss of architecture/necrosis/ intraluminal thrombi
	Histopathologic alteration score

Table 2

Results – histological analysis

The skin segments from both groups had only mild changes during the 14 hour preservation period. There were no signs of edema and/or endothelial cell damage in the VRAM grafts preserved with MP. Figure 15 displays 2 panels of serial biopsies performed in the VRAM grafts' skin during preservation. There is a mild leukocyte infiltration at the dermis and no signs of additional tissue damage during this initial stage.

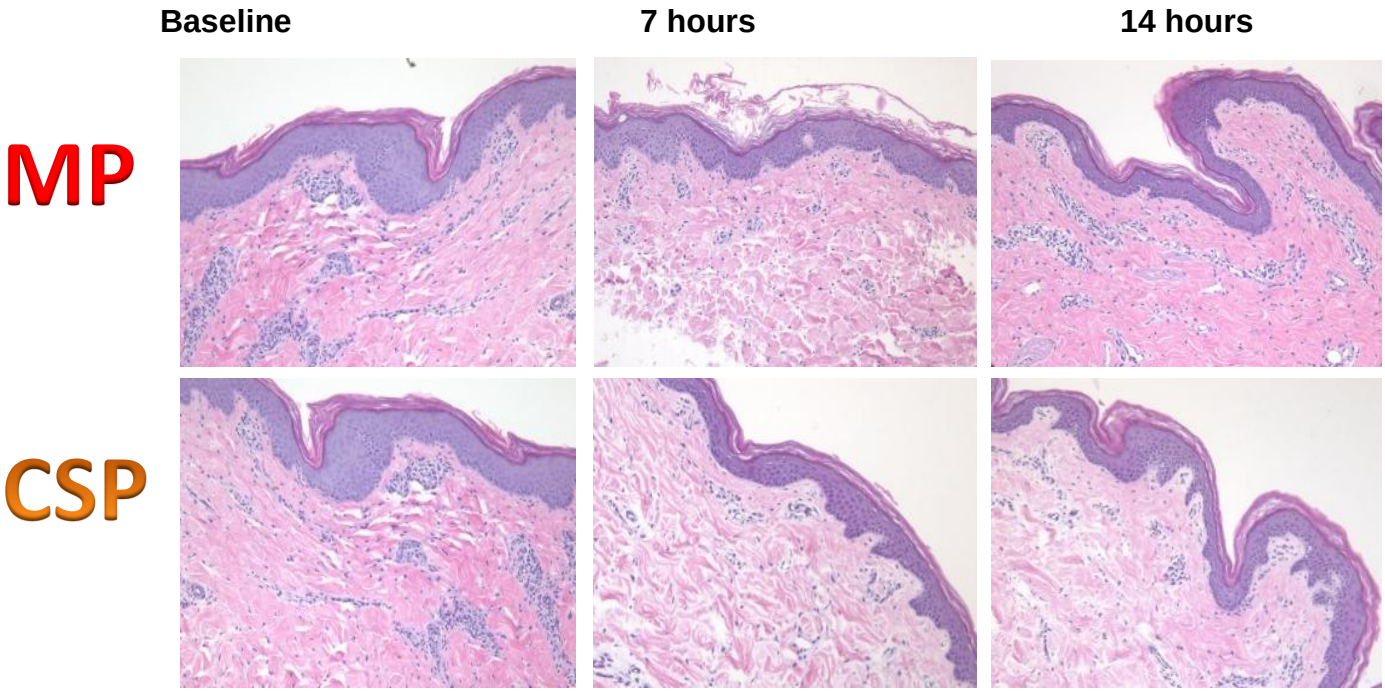


Figure 15

Interesting enough, the muscle segments showed early signs of tissue damage (contraction bands) when the CSP group was compared to the MP group.

The development of hypercontracture and identifiable contraction bands on histology has been extensively studied in the myocardium as a clear and well established sign of early ischemia in striated muscle.

This phenomenon has been previously described [14] and summarized on Figure 16 , where the persistent lack of oxygenation would be the main factor involved in the pathophysiology of this anatomical feature.

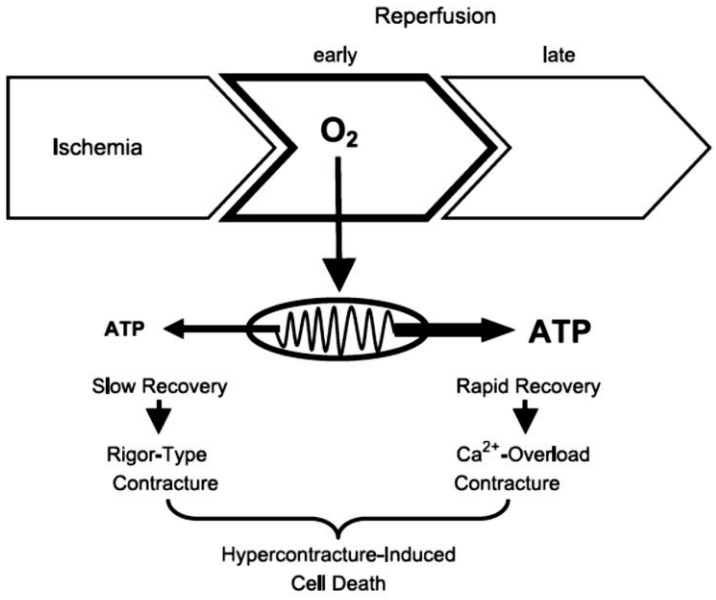
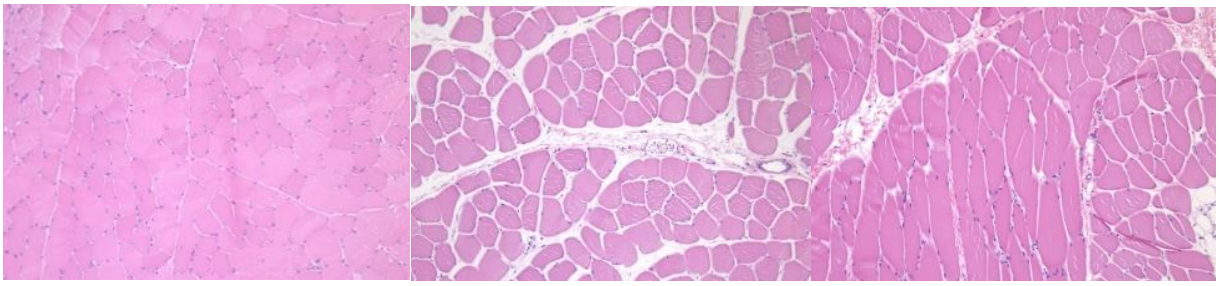


Figure 16

Figure 17 displays panels of serial biopsies in both groups during the 14 hour period for graft preservation. The MP group shows no major histological changes. The CSP group shows early formation of contraction bands within 7 hours and further expansion within 14 hours.

MP Baseline MP 7 hours MP 14 hours



CSP Baseline CSP 7 hours CSP 14 hours

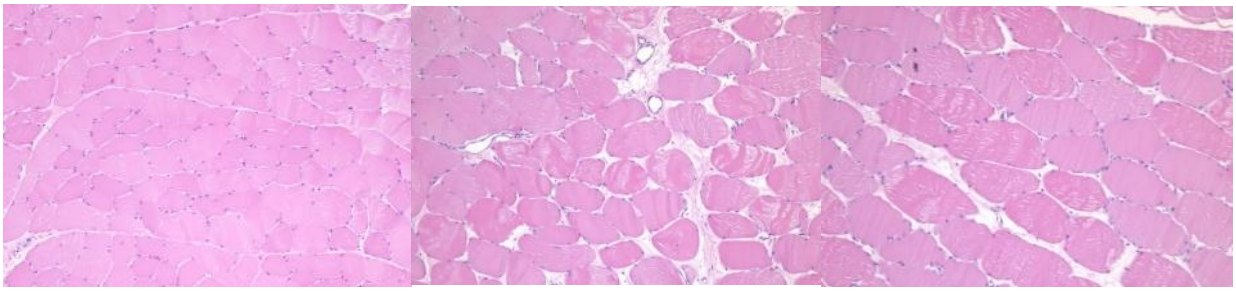


Figure 17

Post-transplantation period

The VRAM grafts were biopsied on days 2, 4 and 7. Early post-reperfusion events were assessed on day 2. It has been clearly demonstrated that the magnitude of the IRIs were more pronounced in the CSP group.

The CSP group, in analogy to the myocardium during ischemic events, showed persistent signs of contraction bands, which became diffuse after VRAM graft reperfusion. There were no histological changes in the MP group on day 2, showing the benefits of the effective oxygenation provided by the MP/HBOC system.

Figure 18 displays a comparison between both groups on the 2nd post-operative day (POD), where the contraction bands initially seem focally at 7 hours in the CSP groups evolved to a diffuse pattern within the muscle tissue.

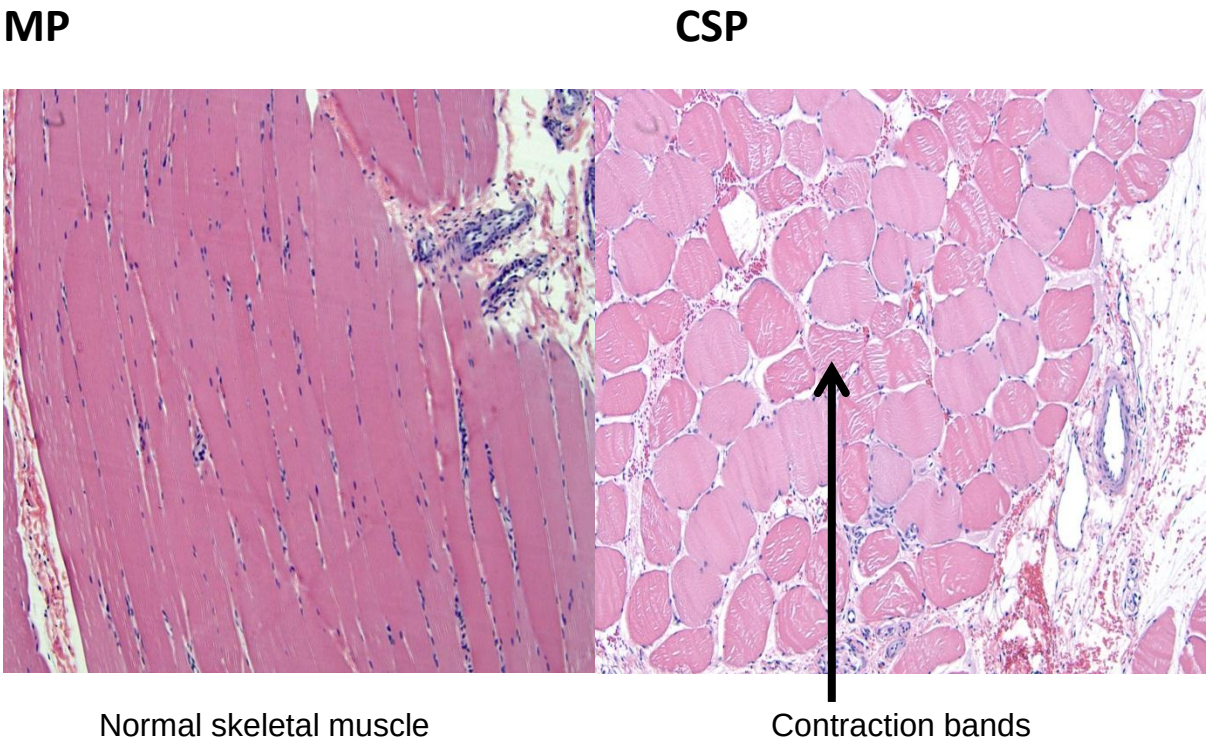


Figure 18

The histological features between the two groups became much more pronounced on the 4th and 7th POD (See Figure 19). The MP group VRAM grafts showed resolution of the mild inflammatory process stemming from none to mild IRIs. The CSP group showed progression of the moderate to severe IRI lesions seen after reperfusion, which were characterized as moderate myocytes ischemic changes and mild interstitial inflammation. The CSP on day 4 displayed features of disseminated degeneration of muscle with diffuse contraction bands. The lack of oxygenation in the CSP resulted in permanent mitochondrial damage and propagation of the Ca^{2+} overload induced contracture leading into further myocyte necrosis.

MP – 4th POD CSP – 4th POD

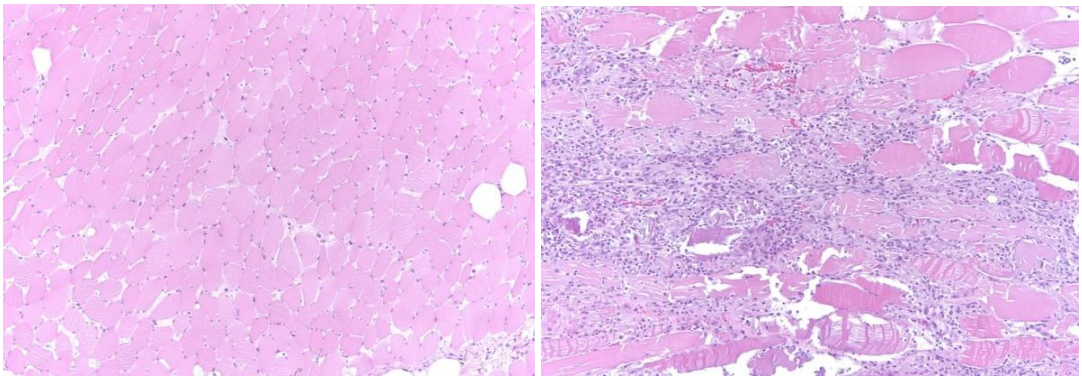


Figure 19

The MP group showed a very mild inflammatory infiltrate within the muscular tissue. The CSP group showed a moderate to severe inflammatory infiltrate composed mainly by lymphocytes and neutrophils. There was exacerbation of the contraction bands and diffuse interstitial edema. Diffuse apoptosis and early necrosis were also seen in the CSP group.

The histological findings on the 7th POD were even more pronounced when the two groups were compared (See Figure 20). The MP group displayed a limited interstitial infiltrate and mild interstitial edema. The CSP group displayed a severe inflammatory infiltrate composed mainly by neutrophils, macrophages and eosinophils. There were diffuse necrosis and several segments of tissue with calcinosis, where histiocytes surrounded wide spread necrotic areas. There was very limited viable muscle tissue on the CSP group on the 7th POD. The same findings were further confirmed by the end-study necropsy.

MP – 7th POD CSP – 7th POD

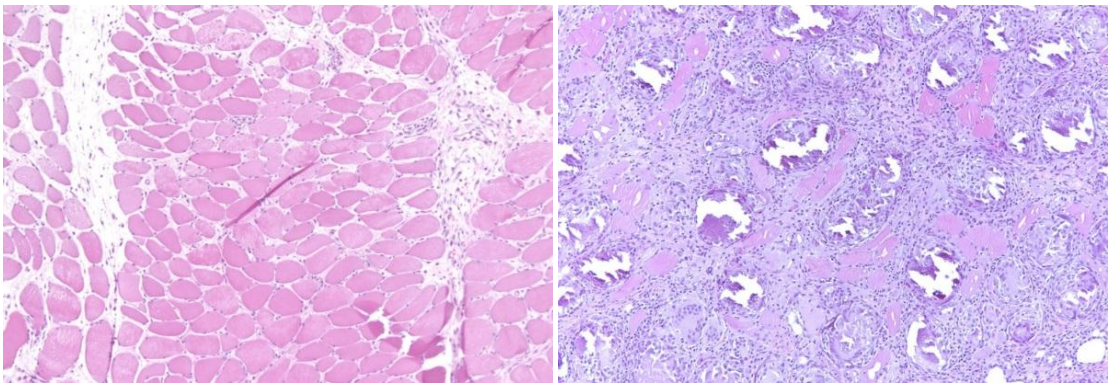


Figure 20

Overall HISS

The HISS were calculated by the combining (mean±SD) the values obtained in each time point (PR= post-reperfusion, 2= 2nd POD, 4= 4th POD and 7=7th POD) of all 3 segments of the VRAM grafts.

Figure 21 displays the HISS scores over the duration of the experiments. The MP group had significantly lower scores across all time points.

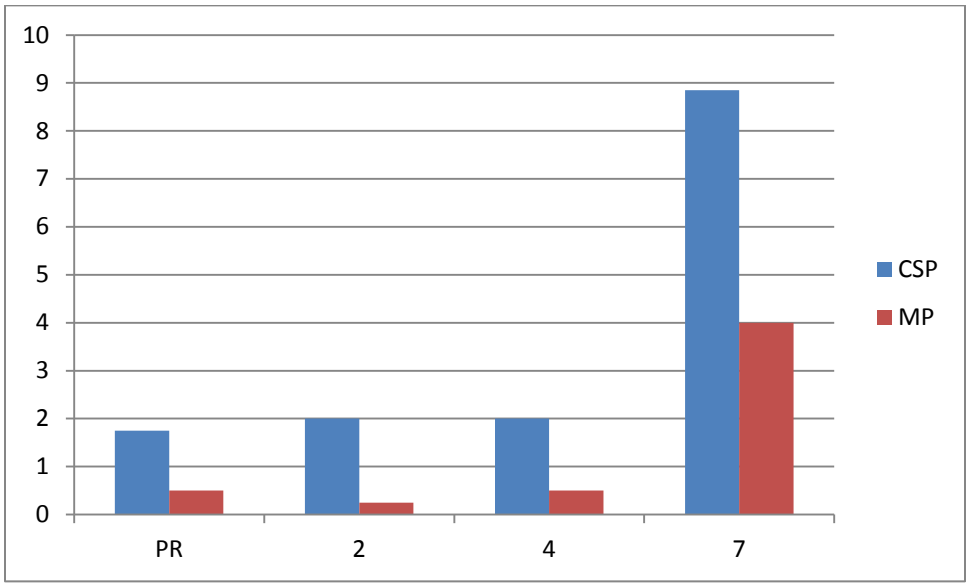


Figure 21

Metabolomics

Tissue samples from the VRAM grafts were obtained during preservation (0, 5, 9 and 14 hours) and after the transplant procedure on POD 0, 2, 4 and 7. These tissues were immediately frozen (OCT) and further submitted to Metabolon Inc., Raleigh, NC for metabolomics analysis. Following receipt, samples were inventoried, and immediately stored at -80°C. At the time of analysis samples were extracted and prepared for analysis using Metabolon's standard solvent extraction method. The extracted samples were split into equal parts for analysis on the GC/MS and LC/MS/MS platforms. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the mass spectrometers. The sample preparation process was carried out using the automated MicroLab STAR® system from Hamilton Company. Recovery standards were added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions; one for analysis by LC and one for analysis by GC. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS. The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a

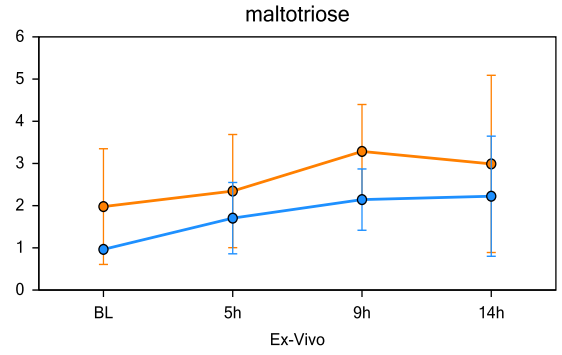
Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% Formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM Ammonium Bicarbonate. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion.

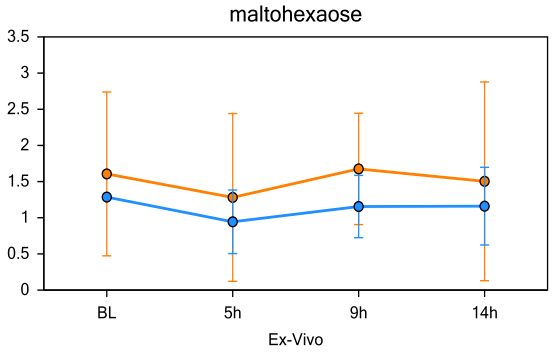
Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the Client Matrix samples, which are technical replicates of pooled client samples.

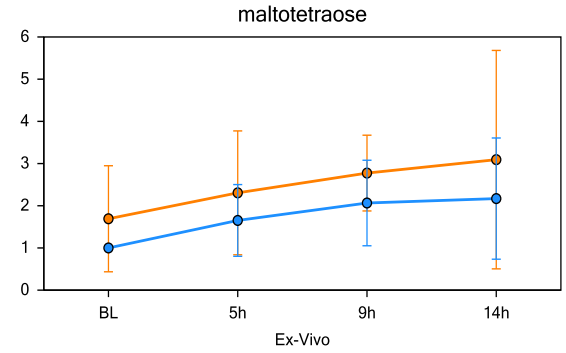
There were 653 compounds analyzed. Following log transformation and imputation of missing values, if any, with the minimum observed value for each compound, Welch's two-sample *t*-tests were used to identify biochemicals that differed significantly between experimental groups. An estimate of the false discovery rate (*q*-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. The *q*-value describes the false discovery rate; a low *q*-value ($q<0.10$) is an indication of high confidence in a result. While a higher *q*-value indicates diminished confidence, it does not necessarily rule out the significance of a result.

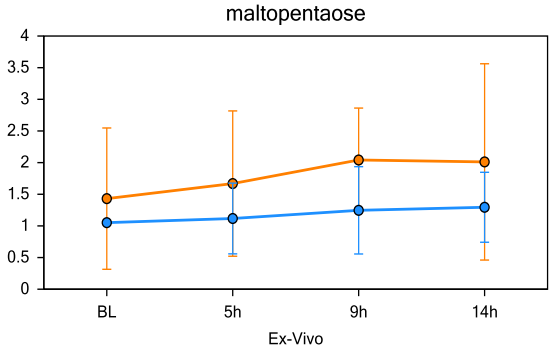
Results

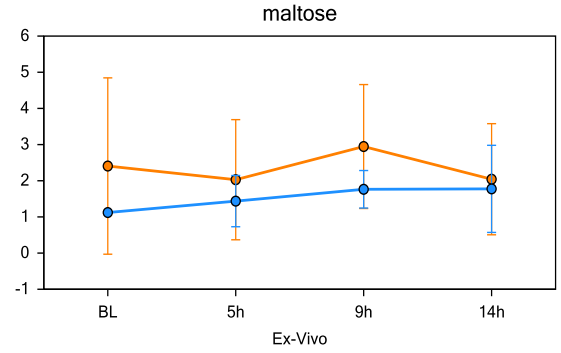
The VRAM grafts in the MP system sustained an intact energetic metabolism, which was mainly fueled by glucose over the 14 hour period of preservation when compared to the CSP group. Glycogen reserves were higher in the MP group. MP compared to CSP showed increases in glycolytic metabolites consistent with a relative increase glucose availability; signs of increasing glycogen breakdown (e.g. increases in maltohexaose, maltopentaose, maltotetraose, maltotriose and maltose, MP vs CSP are consistent with increased glycogen use to support energetics. There was adequate glucose supply in the MP, which was originally provided by the preservation solution. The graphics below represent metabolites levels during preservation. The MP group is represented in yellow (–) and the CSP group is represented in blue (–).



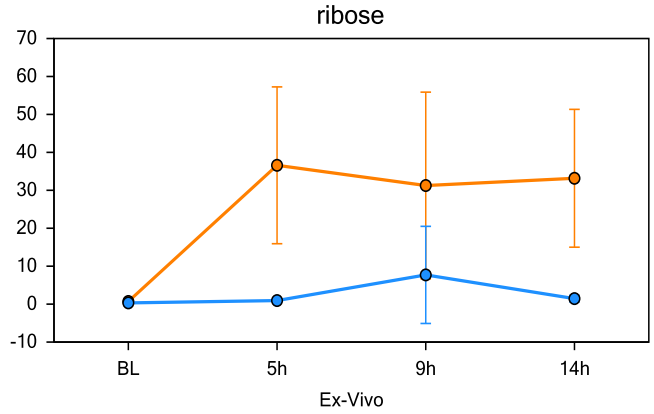

maltohexaose
3.5
3
2.5
2
1.5
1
0.5
0
BL
5h
9h
14h
Ex-Vivo

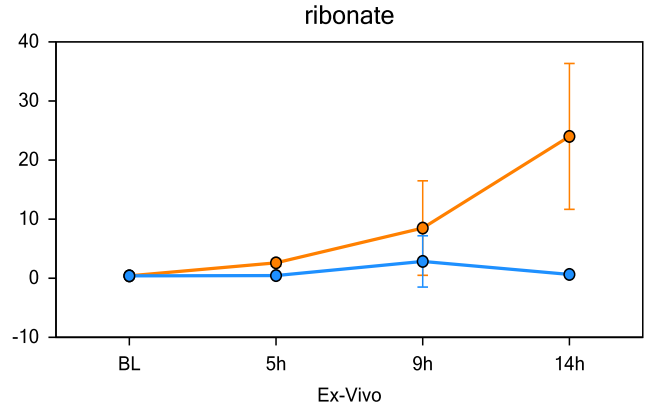

maltotetraose
6
5
4
3
2
1
0
BL
5h
9h
14h
Ex-Vivo

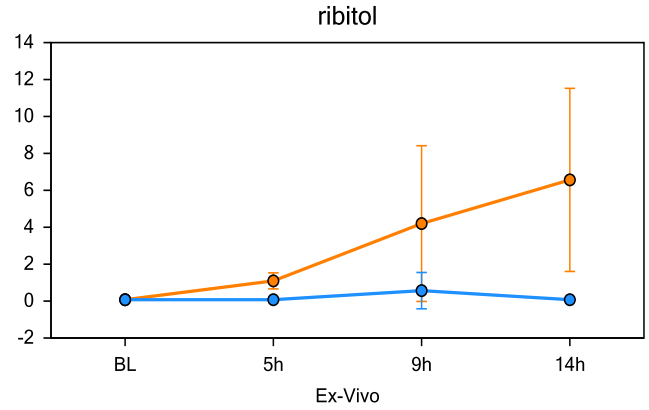

maltopentaose
4
3.5
3
2.5
2
1.5
1
0.5
0
BL
5h
9h
14h
Ex-Vivo


maltose
6
5
4
3
2
1
0
-1
BL
5h
9h
14h
Ex-Vivo

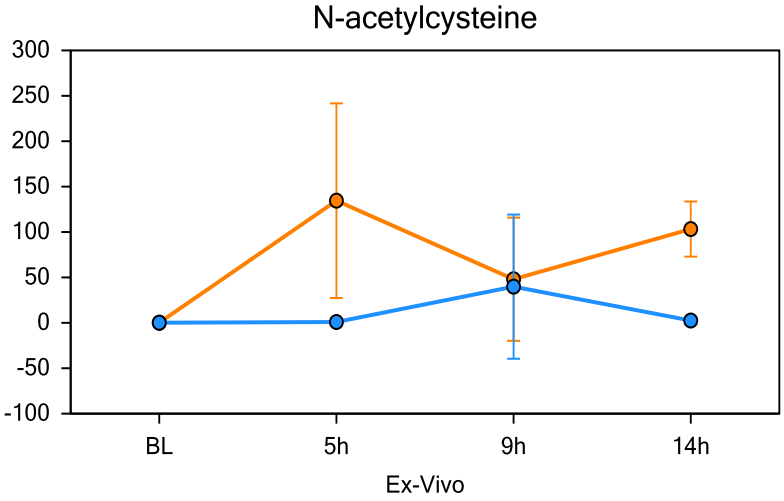
The pentose metabolites were significantly higher in the MP group, showing a higher anabolic state when compared to the CSP group. The CSP group appeared to have a sustained catabolic state when compared to the MP group. There were signs of higher production of nucleotides and nucleic acids precursors in the MP. As previously seen in our experience with liver allografts under the MP/HBOC system, there was a significant (30 fold higher) increase in the metabolic pathways related to cell regeneration once oxygenation was effectively provided ex-vivo during preservation. There were signs of higher production of aromatic amino acids in the MP group when compared to the CSP group.

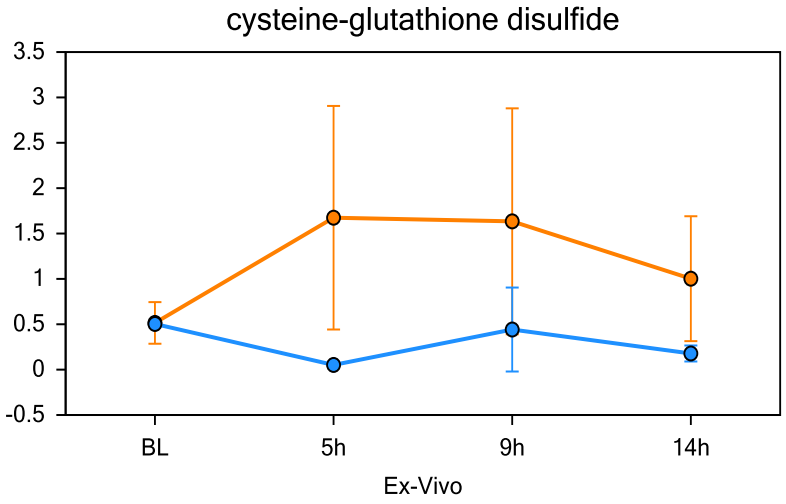




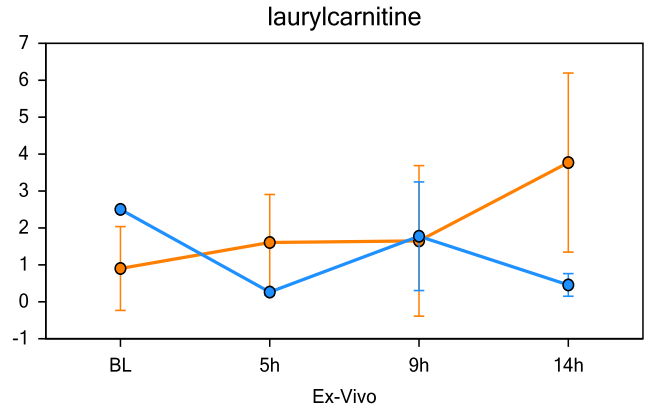


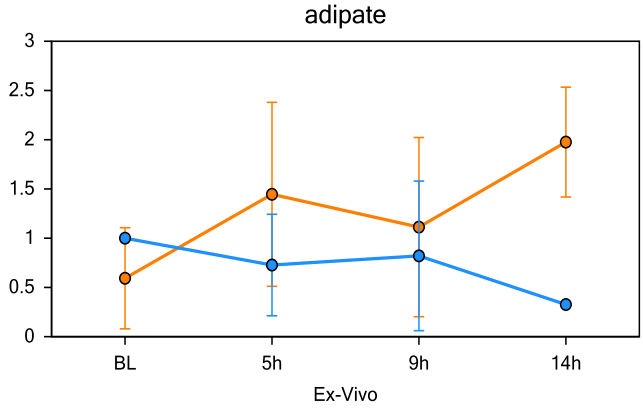
The MP/HBOC system provided more effective anti-oxidant pathways when compared to the CSP group. There were higher levels of end-products from oxidized stress in the MP group, which can be seen as an indirect sign of lower stress from less significant IRIs when compared to the CSP group.

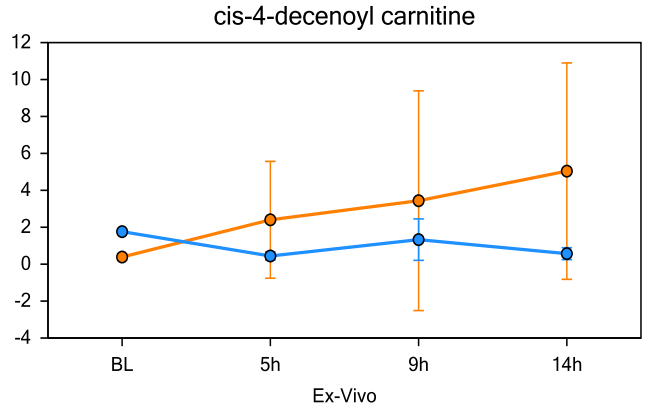


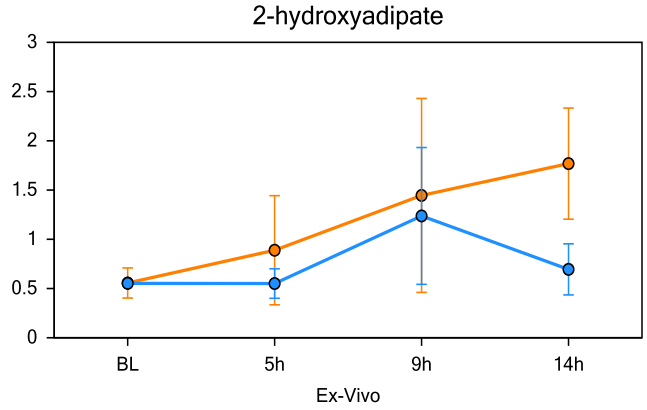


Contrary to our previous experience with livers, the VRAM grafts under the MP/HBOC system showed lower fatty acid β-oxidation when compared to the CSP group. This means a lower amount of fatty acids into the mitochondria as a source of fuel. This also favors our initial findings regarding the preferential pathway for glucose as the primary source of energy in striated muscles.



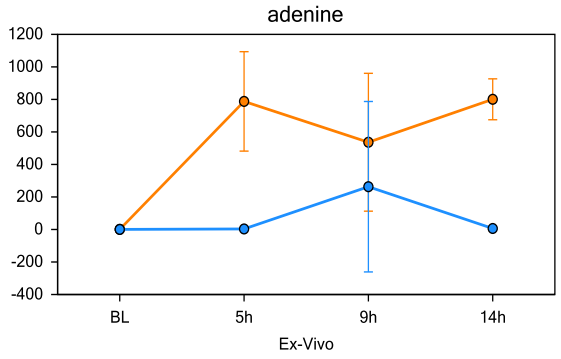


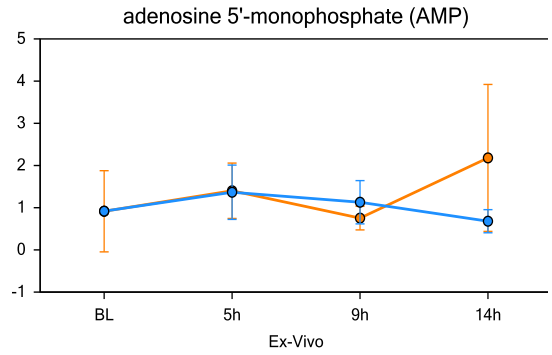


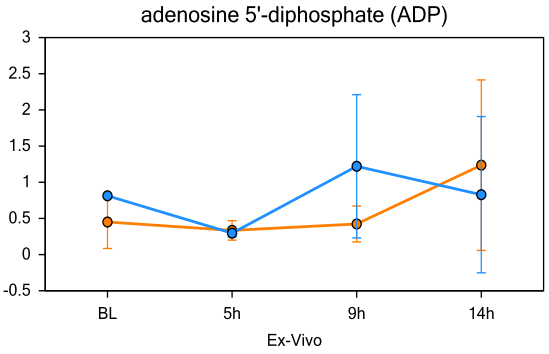


Further analysis of the purine metabolism (adenine components) showed indirect signs of higher ATP production in the MP group when compared to the CSP. The adenine family has a variety of roles in cellular respiration and protein synthesis.

There were higher levels of cAMP in the MP group. AMP is used as a monomer in RNA synthesis. The cAMP as a derivative of ATP has a significant role in signal transduction.




adenosine 5'-monophosphate (AMP)
5
4
3
2
1
0
-1
BL
5h
9h
14h
Ex-Vivo


adenosine 5'-diphosphate (ADP)
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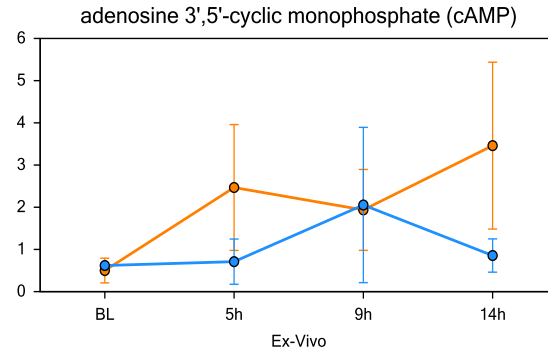
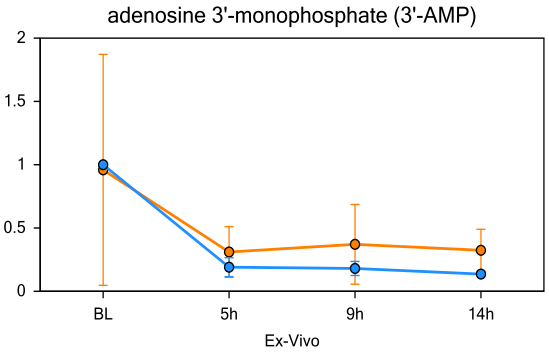
2.5
2
1.5
1
0.5
0
-0.5
BL
5h
9h
14h
Ex-Vivo


adenosine 3',5'-cyclic monophosphate (cAMP)
6
5
4
3
2
1
0
BL
5h
9h
14h
Ex-Vivo


adenosine 3'-monophosphate (3'-AMP)
2
1.5
1
0.5
0
BL
5h
9h
14h
Ex-Vivo

5. Products/Publications/Presentations

A new device for limb perfusion was developed through these experiments. We're able to implement new changes to our original prototype that rendered the filing on a new patent for the new Limb Assist® device to be produced by Organ Assist, Groningen, Netherlands.

The new CTA MP device developed at the MIRM/U Pitt has the following features:

1. The new PVC medical mesh placed horizontally in the limb perfusion chamber (LPC).
2. The VRAM graft placed horizontally and in an anatomic position within the LPC.
3. Free gravity drainage through the PVC mesh from the VRAM graft
4. Pulsatile flow in the infusion port. The changes in the software are specified in a separate section.

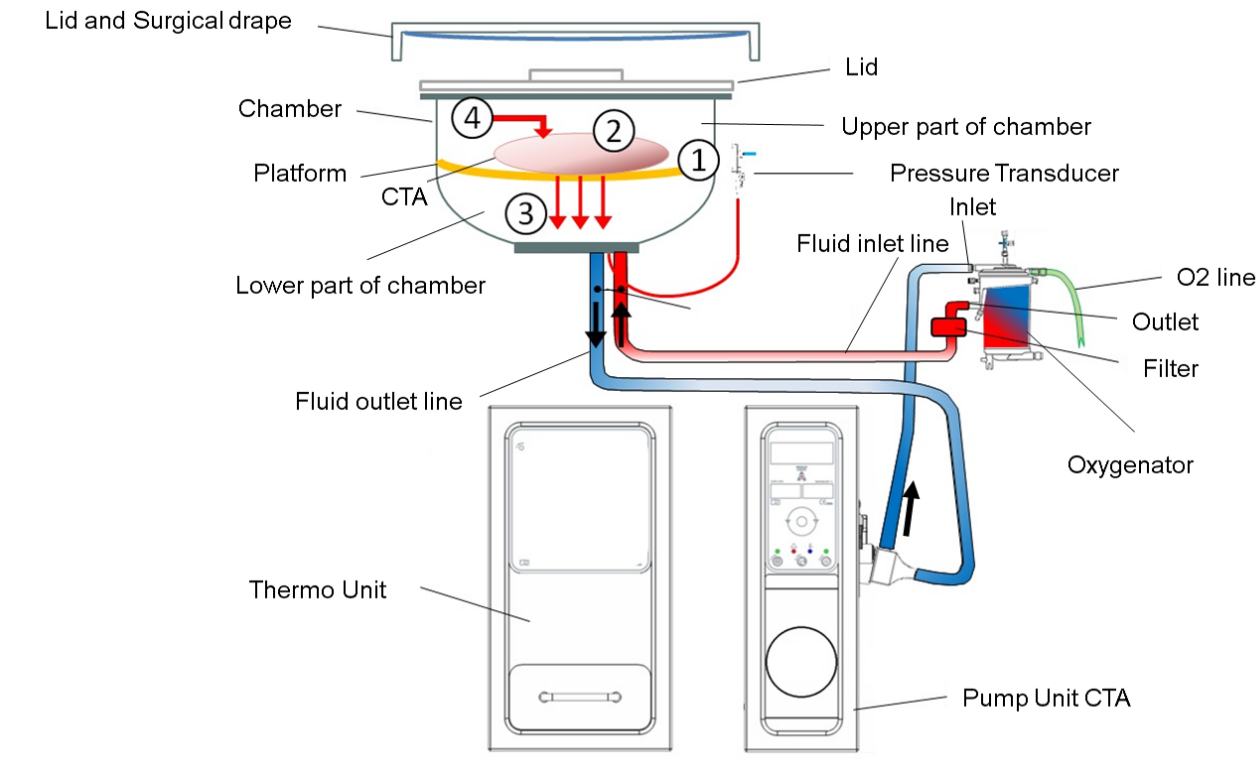


Figure 22

Publications

1. Schweizer R, Oksuz S, Komatsu C, Gorantla VJ, **Fontes PA**. Subnormothermic Machine Perfusion with Hemoglobin-based Oxygen Carriers for Tissue Preservation in Vascularized Composite Allotransplantation. 17th Congress of the European Society for Organ Transplantation, Brussels, Belgium, September 2015.

2. Schweizer R, Oksuz S, Komatsu C, Gorantla VJ, **Fontes PA**. Subnormothermic Machine Perfusion with Hemoglobin-based Oxygen Carriers for Tissue Preservation in Vascularized Composite Allotransplantation. *Jahreskongress Schweizerische Gesellschaft für Plastische, Rekonstruktive und Ästhetische Chirurgie (SGPRAC), Thun, September 11-12 2015.*

3. Schweizer R, Oksuz S, Komatsu C, Gorantla VJ, **Fontes PA**. Subnormothermic Machine Perfusion with Hemoglobin-based Oxygen Carriers for Tissue Preservation in Vascularized Composite Allotransplantation. *Jahreskongress Deutsche Gesellschaft für Plastische, Rekonstruktive und Ästhetische Chirurgie (DGPRAC), Berlin, October 1-3 2015.*

Presentations

1. Invited speaker - 61st Meeting of the American Society of Articial Organs. Perfusing the Kidneys and Tissue Allografts Outside of the Body, Chicago, IL, July 2015

2. Invited speaker – Summer School, McGowan Institute of Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA. The new ex-vivo world; perfusing human organs outside of the body. July 2015

3. Invited speaker – Regenerative Surgery – The Cutting Edge. Organ Preservation: The New Frontier in Organ transplantation. LifeNet Health, Virginia Beach, VA. September 2015.

4. Invited speaker – 1st Annual Meeting of the Society for the Advancement of Transplant Anesthesia, University of Pittsburgh. Organ Preservation Methods. Pittsburgh, PA, September 2015.

6. Inventions/Patents/Licenses

We have developed a new device to optimize subnormothermic (SN) MP in vascularized composite allotransplantation (VCA) by modifying our original Liver Assist Device from Organ Assist, Groningen, Netherlands. A new patent application for the new Limb Assist® device developed under this project has been already filed (attached file #1).

Pitt Ref. No. 03677
Klarquist Ref. No. 8123-95565-01
For **DEVICE FOR COMPOSITE TISSUE ALLOTRANSPLANT PRESERVATION AND USE THEREOF**
Filed September 11, 2015
Provisional Patent Application No. 62217565
EFS ID: 23468151
Confirmation number: 9459
Country: U.S.

7. Reportable Outcomes

These proof-of-concept pre-clinical experiments in a porcine model were very important to determine the role of MP as a new modality for CTA preservation. We're able to reproduce the successful findings published from our liver experience in a similar MP/HBOC system utilized over a 9 hour period.

The MP/HBOC system as a new option for CTA preservation displayed the same safety and effectiveness seen in the solid organ model.

The reported experiments were based on a very challenging model involving 14 hours of CTA preservation followed by graft implantation and the subsequent follow up of the transplant recipients under triple immunosuppressive therapy. The control group VRAM grafts were preserved within the current standard of care (CSP) and showed moderate to severe IRIs with massive necrosis within the first post-operative week.

The MP-treated VRAM grafts had a significantly better outcome, based on the lower magnitude of the IRIs, lower degree of inflammation and additional signs of advantageous metabolic features regarding oxidative stress, fuel utilization and protein synthesis.

The new Limb Assist® device will be utilized on a follow up DOD project (MR140030) led by LtCol Michael Davis, MD, FACS, USAF, MC in the Institute for Surgical Research, San Antonio, TX.

Our specific aims were fully achieved. In summary:

1. The MP/HOBC system promoted an increased CIT period without any damage to the VRAM graft during preservation
2. The MP/HBOC system minimized the IRIs observed during preservation when compared to the current standard of care (CSP).
3. The MP/HBOC system had a positive impact on the immune profile of the VRAM grafts, where a significantly lower degree of inflammation was observed after preservation. This should have a major impact in long term studies where the lesions acquired from IRIs would have a lower degree of alloactivation and most likely a lower degree of both acute and chronic rejection.

8. Other Achievements

The interactions experienced during the data analysis of these experiments have enhanced our links to the ISR, San Antonio, TX and the Department of Plastic Surgery, Zurich, Switzerland. Additional interactions with the Center for Inflammation and Regenerative Modeling (CIRM), University of Pittsburgh, have yielded a great collaborative effort towards the use of computational biology tools for the inflammatory process seen earlier after graft preservation. We intend to proceed with additional studies with the current cytokine data and a further request for this activity will be sent after this report.

We faced a problem with sample storage that further implicated in the quality of the micro array data to be generated from this study. The micro array studies have been dropped and our final budget has a surplus of $46,000 since these tests were not performed by our core lab.

We will implement a further request to utilize this financial resource as a way to fund further analysis to be performed by the CIRM lab at the University of Pittsburgh.

9. References

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2. **Fontes PA**, Marsh W, Lopez R, Soltys K, Scott V, A van der Plaats, W Light, S Shiva, M Minnernvinni, Demetris AJ. Liver preservation with machine perfusion under full oxygenation using a new cell free oxygen carrier solution. American Journal of Transplantation 2013; 13:119.

3. **Fontes PA**, Marsh JW, Lopez RC, van der Plaats A, Light WR, Paranjpe S, Shiva S, Vodovotz Y, Michaolpoulos G. Machine perfusion with a new cell-free oxygen carrier solution provides effective ex-vivo oxygenation, minimizes ischemia reperfusion injuries and downregulates genes associated with liver damage. 16th Congress of the European Society for Organ Transplantation, Vienna, Austria, September 2013.

4. **Fontes PA**, Vodovotz Y, Marsh JW, Lopez RC, van der Plaats A, Light WR, Shiva S, Stolz D, Minnervini M, Barclay D, Handler G, Sadowsky D, Paranjpe S, Michalopoulos G. Ex-vivo hepatic perfusion with a new cell-free oxygen carrier solution upregulates hepatocyte associated gene response against ischemia-reperfusion injury and triggers protective and regenerative pathways. Hepatology 2013, 58; 4(S):211-A.

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10. APPENDICES:

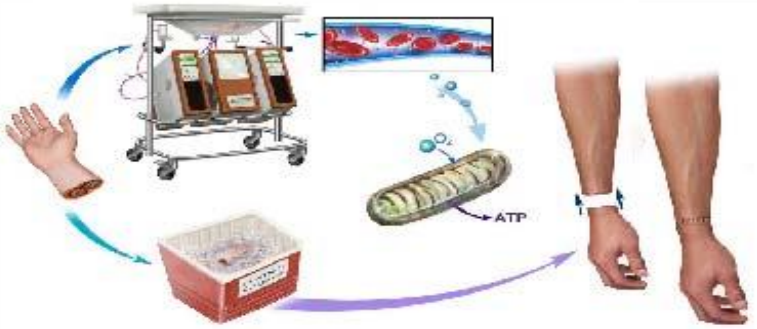
Response to Fund Opportunity No. W81XWH-13-2-0061
Technology Area of Interest: Transplantation
Proposal Title: Ex-vivo machine perfusion in CTA with a novel oxygen carrier system to enhance graft preservation and immunologic outcomes

PI: Paulo Fontes, MD, FACS **Org:** University of Pittsburgh **Award Amount:** $500,000

Objective(s)
This study is specifically designed to improve the functionality of the CTA after transplantation. The central hypothesis is to demonstrate the potential benefits of effective tissue perfusion and oxygenation during the graft preservation period.

Study/Product Aim(s)
- SPECIFIC AIM 1: Determine if the MP-BMPS/HBOC allow prolongation of CIT without significant cellular damage to the allograft.
- SPECIFIC AIM 2: Determine if the MP-BMPS/HBOC minimize the effects and incidence of I/R injury at revascularization.
- SPECIFIC AIM 3: Determine the effect of MP-BMPS/HBOC on the immune profile of various flap tissues after transplantation.

Approach
This study aimed to establish the value of the MP/HBOC system in CTA preservation when compared to the current standard of care (cold static preservation). Ex-vivo and in studies were performed as a way to define the technical issues involved in CTA perfusion and the impact of this technology after graft implantation and subsequent follow up of the transplant recipients under full immunosuppressive therapy.



Machine perfusion with a newly developed cell free oxygen carrier solution as a way to enhance the post-operative function and minimize ischemia reperfusion injuries in CTA. A successful proof-of-concept series of large animal experiments is currently under FDA review for the utilization of this technology as a new preservation modality in liver transplantation.

Timeline and Cost

Activities	CY 13	14	15	16
Infrastructure- Development of research protocol and budget. Organization of personnel and resources.				
Research- testing of perfusion device in various study groups, recording outcomes.				
Data Management – analyze database for trends from study groups.				
Analysis and Reporting – Determine effects on revascularization and immune profile, analyze final data and generate manuscript.				
Estimated Budget ($500K)	**$000**	**$100k**	**$300k**	**$100k**

Goals/Milestones

CY14 Goals
- All surgical experiments have been completed

CY15Goals
- Completed Proteomics analysis
- Completed immunohistochemistry analysis
- Transcriptomics analysis was not performed
- Completed Metabolomics analysis
- Completed histological analysis and incorporate IR score system
- Integrate data analysis with PCA and DBN tools – proposed as next step

CY16 Goals
- Expand data analysis with computational biology

Comments/Challenges/Issues/Concerns – N/A

Budget Expenditure to Date
Projected Expenditure: $500,000; Actual Expenditure: $451,935